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Fermentable Carbohydrate Substrates Generated from Cereal and Pseudocereal Insoluble Dietary Fibers and Their In Vitro Fecal Fermentation

Lisa Marcela Lamothe
Purdue University

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Is approved by the final examining committee:

Bruce R. Hamaker

Osvaldo H. Campanella

Bradley L. Reuhs

John Patterson

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FERMENTABLE CARBOHYDRATE SUBSTRATES GENERATED FROM CEREAL AND PSEUDOCEREAL INSOLUBLE DIETARY FIBERS AND THEIR IN VITRO FECAL FERMENTATION

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For my husband.

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ABSTRACT

Lamothe, Lisa M. Ph.D, Purdue University, December 2014. Fermentable Carbohydrate Substrates Generated from Cereal and Pseudocereal Insoluble Dietary Fibers and their In Vitro Fecal Fermentation. Major Professor: Bruce R. Hamaker.

Dietary fiber has gained an increasing attention in recent years due to the myriad of health benefits attributed to it. The majority of these arise from their fermentative properties and their effects on gut microbiota. Cereals and pseudocereals are important sources of insoluble dietary fibers that are recalcitrant to solubilization by non-chemical methods and poorly fermented. This warranted an effort to make these fibers more susceptible to microbial degradation and a resulting improved fermentability. Insoluble dietary fibers from four alternative grains, sorghum and pearl millet of African origin, and quinoa and amaranth of Andean origin, were subjected to a combination of microwave treatment and sequential enzymatic hydrolysis in order to effect solubilization and improve fermentability. Characterization of the dietary fibers revealed that, besides cellulose and lignin, insoluble fiber from pseudocereals consisted of pectic polysaccharides and xyloglucans, and that arabinoxylans were the main hemicellulose found in cereals. Subjecting insoluble fibers to microwave radiation resulted in low solubilization, but in combination with enzyme hydrolysis solubilization was significantly higher. Quinoa and amaranth were more susceptible to treatments (increase

to ~50% soluble fiber) than pearl millet (increase to ~20 soluble fiber). In addition to solubilization, a portion of the fiber that remained insoluble after treatments became susceptible to microbial degradation and fermentation. Thus, treatments of insoluble fibers generated fiber substrates that were constituted by 3 types of fiber: 1) soluble-fermentable, 2) insoluble-fermentable, and 3) insoluble-nonfermentable. Accordingly, fermentability of treated fiber substrates (TFS) significantly improved to levels higher than predicted solely by increase in soluble fiber content. Improved fermentability was evident in increased total short chain fatty acid production and slow rates of gas production for TFS from pearl millet. Furthermore, fermentation of TFS resulted in significant changes in human fecal microbiota composition following in vitro fermentation. Next-generation sequencing (SBS) of genomic DNA extracted from fecal samples after incubations with TFS showed that the abundance of bacterial groups changed as a response to differences in composition, structure, and degree of fermentability. In general, TFS promoted greater diversity and richness than the single soluble-fermentable control, FOS. In comparison to their untreated counterparts, TFS caused a significant increase in Lachnospiraceae and a decrease in Bacteroidaceae. Substrates from quinoa significantly promoted the Ruminococcaceae family and substrates from pearl millet were more bifidogenic. These results lay the groundwork for the design of fermentable carbohydrates using insoluble fibers to potentially create fiber substrates for improvement of gut health.

INTRODUCTION

For many years, researchers have designed studies that utilize prebiotic oligosaccharides to modulate gut microbiota, because the gut microbiota has been shown that it plays an important role in health and disease (Delzenne et al., 2013; Gibson & Roberfroid, 1995; Rastall et al., 2005; Steer et al., 2000). While researchers continue to study and understand how prebiotics and dietary fibers foster the growth and maintenance of a "healthy" or more remedial microbiota, many have proposed the therapeutic use of single-soluble prebiotic oligosaccharides (e.g., fructooligosaccharides). This is related to the idea that the majority of the health-benefitting properties of dietary fibers are attributed to the fermentation of soluble fibers, and all oligosaccharides are soluble. Here, we refer to single-soluble prebiotic oligosaccharides as a single type of non-digestible oligosaccharide (i.e., FOS, GOS, and XOS) that has been shown to selectively stimulate the growth of specific bacterial groups that are considered beneficial for gut health (Biedryzcka & Bielecka, 2004; Guillón et al., 2014; Rabiu et al., 2001). However, the numerous interactions that occur within the gut microbiota community and the intricacies of their metabolic activity make its modulation via diet a complicated endeavor. In addition, many factors that determine the complexity and variety of fermentable substrates are overlooked in such experimental trials. Overall, single-soluble prebiotic oligosaccharides are readily fermentable substrates that elicit rapid and specific changes

in the composition of the gut microbiota. However, the changes usually involve the promotion of specific bacterial groups thereby decreasing diversity and altering the community's homeostasis. Since it is not yet completely known which specific bacterial species or strains are of significant importance for host-health, caution should be practiced when trying to modulate gut microbiota with single-soluble prebiotic oligosaccharides that promote a small number of bacterial groups.

The interest in adequate dietary fiber consumption stems from studies that provide evidence of an association between a high-fiber diet and reduced risks of the development of metabolic syndrome, gastrointestinal and cardiovascular diseases (Burkitt & Trowell, 1977; De Filippo et al., 2012; Yatsunenko et al., 2012). The high-fiber diets reported in these studies are, given their origin, mainly composed of complex plant polysaccharides. Single, soluble prebiotic oligosaccharides only constitute a minor portion. While single, soluble prebiotic oligosaccharides might serve an important purpose by rapidly promoting a bacterial group that may be depleted in a host with a disease status, a wider variety of fermentable carbohydrate substrates differing in degrees of solubility, fermentability, composition and structure, and physical form are likely better suited for a long-term and sustainable modulation of the gut microbiota.

Insoluble Dietary Fiber

Meta-analyses of the association between diet and the risk of chronic diseases such as obesity, type 2 diabetes, and some cancers report an inverse correlation between the consumption of whole grain and the incidence of these diseases, and it has been shown that there is an independent association with the bran portion of whole grain (Erkkilä et al., 2005; Jensen et al., 2004; Koh-Banerjee et al., 2004). The bran fractions of cereal and pseudocereal grains often is concentrated in insoluble dietary fiber and is composed of cell-wall polysaccharides (cellulose and hemicelluloses), lignin, protein, and phenolic acids (Vitaglione et al., 2008). Since the insoluble dietary fiber fraction contained in bran is composed of a variety of cell-wall polysaccharides, increasing its fermentability can produce fermentable fiber substrates that provide both fermentative and non-fermentative health-benefitting properties. In addition, they can also be used as sources for a variety of oligo- and polysaccharide structures that can potentially support the growth of a wider and more diverse group of microbial species.

Hypothesis and Specific Objectives

The use of dietary fibers from the grain sources of quinoa, amaranth, pearl millet, and sorghum, that are not prevalent in the Western diet, can provide different forms or types of fibers, and increasing their fermentability through physical pretreatments may be a way to make them more nutritionally functional. This is explored in this project. The first objective of this thesis work was to quantify the soluble and insoluble fractions, and to partially characterize the chemical structures, of dietary fibers from these four alternative grains of Andean and African origin (quinoa, amaranth, pearl millet, sorghum)

(Chapter 2). The second objective was to develop a non-chemical treatment, or series of treatments, that can effectively solubilize and/or modify the insoluble dietary fiber portion found in the alternative grains (Chapter 3); and to improve its fermentability, as measured by increasing their SCFA production, as well as their propiogenic and butyrogenic properties (Chapter 4). The final objective of this thesis was to evaluate the effect that the fermentable carbohydrate substrates, generated by the treatments, had on the composition of the fecal microbiota community using *in vitro* fecal fermentation (Chapter 5).

CHAPTER 1. LITERATURE REVIEW

1.1 Impact of Gut Microbiota on Health and Disease

The vast number and variety of microorganisms that reside in the human gut constitute a complex community whose members interact among themselves and with the host, thereby significantly impacting health and physiology (Clemente et al., 2012). Such is the importance of the gut microbiota that mammals have evolved to assist colonization of commensal microbes for their own development and contribution to a healthy microbiota. Autochthonous bacteria in the gut provide the host with essential vitamins, metabolism of indigestible compounds, defense against pathogenic invasion, and play a role in the development of the intestinal epithelium and immune system (Hooper et al., 2000; Mazmanian et al., 2014)

Gut microbiota also provide an important role in the development of the intestinal epithelium. Comparisons between germ-free and conventional animals have revealed that the former have a thinner mucus layer (Sharma et al., 1995), a smaller intestinal surface (Gordon & Bruckner-Kardoss, 1961), an impaired brush border differentiation (Abrams et al., 1963), a reduced villus thickness (Reinhardt et al., 2012), and an under-developed immune system (Round & Mazmanian, 2009). The mucosal immune system operates in the intestinal tract and fulfills two very distinct functions; it imparts tolerance

of the commensal microbiota and also protects the intestine from the overgrowth and/or penetration of pathobionts[1] (Sommer & Bäckhed, 2013). The interaction between the immune system and gut microbes are crucial for gut homeostasis (Rakoff-Nahoum et al., 2004) and this is best exemplified by studies on the gut microbiota and inflammatory bowel disease (IBD). IBD, which is characterized by exacerbated inflammation in the gastrointestinal tract, involves an unrestrained pro-inflammatory immune response that is mediated by the interaction of intestinal bacteria with the immune system (Simpson et al., 2000). Since different bacterial groups can elicit specific immune responses or confer different functionality, the composition of the gut microbiota is an important factor for the induction of disease (Frank et al., 2007; Muegge et al., 2011; Turnbaugh et al., 2008). Moreover, metabolic processes encoded in the gut microbiome are intricately connected with the host's metabolism of many nutrients (Nicholson et al., 2005). For example, the microbiome has been shown to modulate the metabolism of dietary lipids (Martin et al., 2007). Accordingly, metabolic patterns of the gut microbiota have also been associated with the onset of insulin resistance, low-grade inflammation, non-alcoholic fatty liver disease, obesity, and diabetes (Aron-Wisnewsky et al., 2013; Cani et al., 2008; Zhang et al., 2010a). Given the significant involvement of the gut microbiota in human health and the development of disease, it is important to investigate effective ways of modifying its composition. From a carbohydrate chemist's vantage point, modulation of the gut microbiota can be achieved by means of fermentable carbohydrate substrates that result in targeted changes according to the substrate physicochemical characteristics.

[1] Pathobiont: a harmless microorganism that can become pathogenic under certain environmental conditions.

1.2 Dietary Fiber

It is widely recognized that dietary fiber plays an important role in many physiological functions and in the prevention and treatment of certain diseases such as inflammatory bowel disease and colon cancer (Rose et al., 2007). Cereals remain the main source of energy in the diets of traditional agrarian societies; and importantly, many are consumed in an unrefined form that results in increased intake of dietary fiber (Collins et al., 2010). Westernized diets that are deficient in dietary fibers and include a significant amount of refined carbohydrates and saturated fats have been associated with increased incidence of gastrointestinal diseases, obesity, high blood pressure, diabetes and cardiovascular disease (Gemen et al., 2011). Over the years, a large number of studies have described the many physiological functions and outcomes of dietary fiber. For instance, some fibers have the capacity to bind bile acids which increases their release in the feces. This, in turn, increases the requirement of cholesterol for the production of more bile acids which results in a decrease of serum cholesterol levels that is related to a reduced risk of cardiovascular disease (Rose et al., 2007). Increased dietary fiber intake has been associated with a reduction in blood pressure in patients with hypertension (Whelton et al., 2005). While the potential mechanism is not well defined, it may be related to the ability of dietary fiber to enhance insulin sensitivity; insulin may play a role in blood pressure regulation (Landsberg, 2001; Streppel et al., 2005). Dietary fiber also appears to have potential for the prevention of type 2 diabetes (Kaline et al., 2007); the consumption of sufficient dietary fiber levels out postprandial glycemic and insulinemic responses and favorably influences level of plasma lipids in type II diabetes patients (Tabatabai & Li, 2000). It has also been suggested that dietary fiber reduces the risk of weight gain

leading to obesity (Papathanasopoulos & Camilleri, 2010). Therefore, dietary fibers have been at the forefront in the list of functional foods that promote colonic and overall health (Hijova & Chmelarova, 2007) and the fermentable dietary fibers are of specific interest.

The major products of the bacterial fermentation of dietary fiber in the colon are short chain fatty acids (SCFAs) of which butyrate and propionate are of importance since it has been suggested that they have a role in metabolic and inflammatory disorders (Puertollano et al., 2014). Butyrate is an energy source for epithelial cells and influences cellular functions related to anti-carcinogenic and anti-inflammatory responses, thus suggesting a protective role against colonic diseases such as colorectal cancer and inflammatory bowel disease (Hamer et al., 2007), although conclusive evidence of this protective effect is still lacking. Propionate has been shown to increase satiety by increasing leptin production, to inhibit the formation of pro-inflammatory cytokines in human adipose tissue, lower blood glucose and alter lipid metabolism (Al-Lahham et al., 2010; Curi et al., 1993; Hinnebusch et al., 2002; Todesco et al., 1991; Xiong et al., 2004). Thus, the identification and/or development of fiber substrates that promote the production of butyrate and propionate are of interest.

1.2.1 Dietary Fibers as Food Ingredients

Evidence suggesting the beneficial effects of dietary fiber on human health has prompted food scientists to design and develop food products with higher dietary fiber content that appeal to consumers' interest of adopting healthier eating habits. Studies have shown that dietary fiber consumption in the United States is significantly less than recommended levels. This is due, in large part, to the fact that the majority of commonly consumed

foods have low dietary fiber content. Food products made from whole grain or that are fortified with fiber-based ingredients often have low acceptance among some consumers due to either real or perceived undesirable sensory quality. An important factor to consider is that specific physicochemical and colonic fermentation characteristics of dietary fibers will determine their functionality and how they can be used or incorporated into food products. For example, the insoluble fraction of cereal dietary fibers presents significant difficulties for its incorporation in food products; however, they can be used as sources of fiber substrates with improved functionality and fermentability. Furthermore, a current trend to find new sources of dietary fibers has resulted in research efforts that investigate the potential use of agronomic and/or cereal processing by-products as materials that can yield a wide range of fiber substrates (Rodríguez et al., 2006).

1.2.2 Insoluble Dietary Fibers as Sources for Fermentable Fiber Substrates

Cereals are good sources of insoluble dietary fiber that is mainly composed by arabinoxylans, cellulose, lignin, structural proteins and esterified phenolics (Selvendran, 1984). Arabinoxylans are the predominant nonstarch polysaccharides in fibers from cereals such as wheat, maize, sorghum and pearl millet and they differ in the amount, structure, solubility and molecular weight according to the genetic makeup of the cereal species and the environmental conditions of the grain development (Collins et al., 2010; Crittenden et al., 2002). Arabinoxylans consist of a β-(1-4)-linked D-xylose backbone that is substituted on O-2 and/or O-3 by side chains that may be composed of single arabinose units or by more than one sugar residue like arabinose, xylose and/or galactose

(Izydorczyk & Biliaderis, 1995). Other substituents of the xylan backbone include glucuronic acid (Chanliaud et al., 1996) and ferulic acid (Dervilly et al., 2000). They are commonly classified into two groups; water-extractable and water-unextractable arabinoxylans that can be solubilized in alkaline solutions and/or by enzymatic degradation (Arrigoni, 2001). In general, water-unextractable arabinoxylans pass through the colon largely undigested and their major contribution to colonic health is due to non-fermentative effects. On the other hand, water-extractable arabinoxylans, as found in wheat (principally endosperm) and other similar grains, usually ferment at a fairly faster rate, and much faster than less soluble non-starch polysaccharides. Because they are fairly rapidly fermented, water-extractable arabinoxylans leave low quantities of carbohydrate substrate for bacteria in the distal colon (Cummings et al., 2001). Solubility of nonstarch polysaccharides is affected, to a large degree, by their crosslinks and other interactions with other constituents in plant cell walls. According to van Laar et al. (2002), arabinoxylans form a large network throughout the cell wall by hydrogen bonding with cellulose and esterified crosslinks with ferulic acid. This same group of researchers studies the fermentation rate of maize arabinoxylans that were solubilized by mild alkali treatment and concluded that breaking the interactions among arabinoxylans with other constituents in the maize cell wall increases their fermentability.

Considering that cereal dietary fiber generally exhibits low solubility in water, they are not ideal for short-chain fatty acid production in the colon. Indeed, cereal dietary fibers are by and large poorly fermented given that the majority of their dietary fiber content is insoluble and not susceptible to microbial degradation. However, properties of cereal

dietary fiber can be modified through certain types of treatments that include physical and practical enzymatic processes. In addition, current research has shown that non-chemical methods such as hydrothermal treatments can effectively solubilize hemicelluloses from insoluble dietary fibers (Pronyk et al., 2011; Roos et al., 2009; Rose & Inglett, 2010a). Treating cereal dietary fibers in order to increase their solubility has been shown to improve fermentation properties. Hydrothermal processes such as extrusion and autoclaving affect dietary fiber in terms of their physiological properties because the mechanical treatment and heat disorganize the original structure of the raw fiber molecules (Björck & Asp, 1984). As previously mentioned, cereals are good sources of insoluble dietary fibers. Based on the enzymatic-gravimetric method, insoluble fiber content on cereal whole grain flours has been reported to be ~11% for wheat and maize (Björck & Asp, 1984; Picolli da Silva & Ciocca, 2005). The amount of insoluble fraction in sorghum dietary fiber has been reported to range from 6.5-8% (Dendy, 1995), but other studies have reported higher insoluble fiber contents of sorghum such as that of Picolli da Silva & Ciocca (2005) who indicated an 11% average content. For pearl millet, 13-14% of its total dietary fiber is insoluble (Raggae et al., 2006).

The growing interest in alternative grains due to increased awareness of the beneficial effects of dietary fibers has led to the study of other sources of nonstarch polysaccharides that may have different or better functional properties and health benefits. Alternative cereal sources that have been investigated include sorghum and pearl millet, which lack gluten and are widely consumed in Africa and Asia. Sources different from cereals

include quinoa and amaranth that are referred to as pseudocereals because they are the seeds of dicotyledonous species but are consumed in the same way as cereal grains.

1.2.2.1 Sorghum (*Sorghum bicolor*)

Most of the dietary fiber in sorghum is insoluble and arabinoxylans are the major component (Verbruggen et al., 1993). The functional properties of sorghum nonstarch polysaccharides have not been extensively studied but given that the majority are insoluble, it is unlikely that they behave as hydrocolloids in food systems like endosperm wheat arabinoxylans do (Huisman & Voragen, 2000; Muralikrishna & Subba Rao, 2007). Therefore, sorghum nonstarch polysaccharides are not sufficiently functional by themselves in order to generate good-quality baked products (Taylor & Naushad, 2010). In addition, it has not been reported that sorghum insoluble dietary fiber has specific or unique health-promoting properties other than increased bowel movement, softer stools and increased stool weight, which are ascribed to the insoluble dietary fibers in general (Cornu & Delpeuch, 1981; Taylor & Naushad, 2010). Accordingly, it is of relevance to attempt to modify the solubility characteristics of sorghum dietary fiber given that it is a staple crop in many African and Asian countries. Even though sorghum is normally decorticated (debranned) prior to milling into flour and preparing foods, the high level of consumption of sorghum and its products justify the need to employ this cereal source as a whole grain, high fiber food for the physiological benefits of dietary fiber. Increasing fermentable fiber content would enhance the health-related properties of sorghum, as well as likely result in improved functional properties that would allow its use in a greater variety of food products.

1.2.2.2 Pearl Millet (*Pennisetum glaucum*)

The millets are another important cereal crop in African and Asian arid and semi-arid regions since they are well adapted to dry, sandy and acidic soils characterized by low fertility. The millets are nutritionally equal or superior to other cereals (Obilana & Manyasa, 2002). The species consists of two broad categories; the minor millets that include finger, proso and foxtail millet as well as tef and fonio, and the major category that is pearl millet (*Pennisetum glaucum*), which is the most widely grown. Pearl millet is highly nutritious; it is rich in methionine and cysteine and has a fat content that ranges from 3-7%, which is higher than in most common cereals (Abdalla et al., 1998). Dietary fiber content of pearl millet has been reported to range from 17% to 20% (Kamath & Belavady, 1980; Singh et al., 1987). Much like sorghum, dietary fiber of pearl millet is mostly insoluble (Dendy, 1995). Decortication of pearl millet removes approximately 7.5% of the grain yielded bran fractions that contained about 38% insoluble fiber and 1% soluble fiber (Rooney et al., 1992). In that same study, the pearl millet bran fraction was fed to rats and it had high bulking capacity, but only slightly reduced serum cholesterol levels which is consistent with the insoluble-soluble dietary fiber profile of the pearl millet bran previously described. Bailey, Sumrell and Burton (1979) found that pearl millet pentosans are composed of seven sugars of which the most predominant are arabinose, xylose, glucose and galactose. Additionally, Hadimani et al. (2001) reported uronic acid content in pearl millet nonstarch polysaccharides. Further reports of the structural features of pearl millet nonstarch polysaccharides are scarce. Nandini and Salimath (2002) reported that arabinose and xylose residues constituted an 82% of the total sugars, approximately 10% was represented by galactose and glucose and 7% was

uronic acid. Methylation analysis of this barium hydroxide-extracted arabinoxylan fraction indicated that the main chain is composed of β(1→4) linked xylose units with substitutions of arabinofuranosyl residues at the O-3 position. Most of the arabinose units were present as terminal sugars and 5% of them were branched. This grain has shown very good potential to increase nutritional value of a variety of food products. Considering its high amount of dietary fiber, there is interest to further study its potential as a good source of nonstarch polysaccharides in the diets of African and Asians and as a new fiber fortification ingredient for food products from around the world. As with sorghum, the insoluble nature of its dietary fiber creates the possibility for enhancing the soluble fraction of these polysaccharides to improve functionality and nutritional benefit.

1.2.2.3 Amaranth (*Amaranthus caudatus* L.)

Amaranth is an ancient grain of the American continent that is still traditionally grown today in some areas of South America. It is native to the Andes of Peru, Ecuador, Bolivia and Argentina. The use of this grain has become popular in specialty foods which has led to an increase in its production in regions of the United States, Canada, Europe and New Zealand. The uses given to amaranth in these South American countries include whole grain consumption, leaf vegetable and for medicinal purposes (Williams & Brenner, 1995). Many amaranth species are considered weeds and only three species that produce light-colored seeds are used for human consumption; *Amaranthus hypochondriacus, Amaranthus caudatus,* and *Amaranthus cruentus* (Espitia-Rangel, 1994). According to a summary of amaranth chemical composition data, its dietary fiber content can range from 8.0 to 16.0% (Berghofer & Schoenlechner, 2002). Amaranth differs from cereals in the

proportion of soluble dietary fiber from the cereals above; 33.0-44.0% of the total dietary fiber content of pale-seeded varieties of amaranth is soluble which is significantly greater than the proportion of soluble dietary fiber in cereals like sorghum, wheat and maize (Pedersen et al., 1990). The monosaccharide composition of amaranth dietary fiber also shows that it differs from that of the common cereals grains. Comparatively, the amaranths have a lower content of xylose and glucose, but higher uronic acid content. Klason lignin content tends to be high even in pale-seeded varieties (Nyman et al., 1984). Amaranth also has higher cellulose content than maize, millet, rice or sorghum (Wang et al., 1991).

Amaranth is a dicotyledonous, small grain-producing species that differs from monocotyledonous species in cell wall compositional characteristics. The primary cell walls of dicotyledonous species are rich in pectins, xyloglucans and cellulose. Ferulic acid is commonly found ester-linked to pectins (Bunzel et al., 2005) and amaranth also contains phytic acid. Repo-Carrasco-Valencia et al. (2009a) reported a content that ranged around 0.3%. The phytic acid content of amaranth is low compared to common cereals like maize and wheat (Guzman-Maldonado & Paredez-Lopez, 1998); for instance, wheat bran has a phytic acid content of around 5% (Gualberto et al., 1997). Lorenz and Wright (1984) concluded that phytic acid is distributed throughout the amaranth seed, because a reduction was not evident after dehulling, tannins on the other hand, were reduced by 80%. In general, the total content of phenolic compounds in amaranth has been reported to be greater than in oats, wheat, maize and sorghum (Repo-Carrasco-Valencia et al., 2009a). There is a need to study the dietary fiber fraction of amaranth

grains beyond total, insoluble and soluble dietary fiber quantification. Few studies have reported in depth analysis of dietary fiber quantification as well as monosaccharide composition and linkage analysis of amaranth dietary fiber. It is of relevance to further study the physicochemical characteristics such as detailed monosaccharide composition, degree of branching, degree of substitution, and substituent characteristics, due to its good potential as a functional ingredient as well as potential physiological benefits that this pseudocereal may provide.

1.2.2.4 Quinoa (*Chenopodium quinoa* W.)

Another important pseudocereal that has gained popularity in recent years is quinoa (*Chenopodium quinoa* Willd.). It is known in particular for its high protein quality and good nutritional value (Ranhotra et al., 1993). In the pre-Columbian Incan culture, quinoa was an important crop and it was referred to as "the mother grain" (Abugoch, 2009). Like amaranth, quinoa is a dicotyledonous, annual plant that is mainly found in the Andean region of Bolivia, Ecuador and Peru. Recently, supply of quinoa has not been sufficient to meet growing demands of markets in the United States, Europe and Asia (Jacobsen, 2003). Thus, it is currently also cultivated in China, Europe, Canada, India and the United States. Quinoa seeds are oval-shaped, flat and the most common varieties produce seeds of pale-yellow color. An important characteristic of quinoa seeds is a pericarp rich in saponins. These compounds generate a bitter flavor and have an anti-nutritional effect that requires that the seeds be washed before consumption.

Ranhotra et al. (1993) stated that the total dietary fiber content of quinoa is in a similar range as found in cereal grains and leguminous seeds. According to Ruales (1994),

washed quinoa contains around 13% total dietary fiber. However, this amount may vary as it has been reported in other studies to range from 7-10% total dietary fiber content (USDA, 2005). Up to 70% of the total dietary fiber content is insoluble and the majority is found in the seed pericarp (Ando et al., 2002). It appears that the pericarp of quinoa seeds contains the majority of its protein content, since the bran of quinoa seeds accounts for 65% of the total protein (Chauhan et al., 1992). It is of relevance to investigate the linkages between protein and polysaccharides in quinoa bran. As it has been suggested by Saulnier et al., (1995), the potential protein-polysaccharide interactions may be a cause for dietary fiber insolubility.

Altogether, quinoa has proven to be an interesting alternative in food product development. Studies on the effect of extrusion cooking of quinoa have reported that the insoluble fraction of quinoa dietary fiber tends to decrease and the soluble fraction increases. According to speculation by Gualberto et al. (1997), this is probably due to the breakage of chemical bonds of insoluble dietary fiber molecules caused by high temperature and high screw speed to which the sample is subjected during extrusion cooking. The breaking of chemical bonds results in smaller particles that have a more soluble nature. Quinoa's potential as a food source has been mainly attributed to its high protein quality, but studies that quantify and/or characterize its dietary fiber are uncommon. As stated before, quinoa's dietary fiber content is comparable to that of cereal grains but very little is known about its physicochemical characteristics and nutritional benefits. Like amaranth, quinoa is not a true cereal and the monosaccharide profile and linkage patterns of its nonstarch polysaccharides might differ from that of

cereal grains. These differences may result in different and interesting functional properties and colonic fermentation patterns. As the demand for this pseudocereal grows, it is important to understand specific physicochemical properties of quinoa dietary fiber to elucidate potential new physiological and functional benefits of quinoa seeds. As mentioned above, because quinoa dietary fiber is mainly insoluble, an attempt to increase its soluble fraction may have good applicability to improve its nutritional and functional properties. However, it should be noted that heat treatments such as cooking and autoclaving have been shown to significantly reduce the amount of soluble dietary fiber in quinoa foods (Ruales et al., 1994). The researchers concluded that some soluble fiber was lost during cooking and autoclaving seemed to cause fiber aggregation. Further research should aim to systematically understand the effect of processing methodologies on quinoa nutritional and functional properties.

1.2.3 Modifications of Insoluble Dietary fibers to Generate Fermentable Carbohydrate Substrates

The benefits of dietary fiber are considered to be of two types; the first is the non-fermentative properties which result in increased fecal bulk, decreased colonic transit time, carcinogen and bile salt binding and increased digesta viscosity. The second type refers to its fermentative properties which result in the production of short-chain fatty acids that have physiological functions in the colon (Rose et al., 2007) and of potential beneficial changes in microbiota. McBurney and Thompson (1992) stated that the effects of dietary fibers in the colon depend, to some extent, on their susceptibility to bacterial fermentation. The specific characteristics of polysaccharides that reach the colon such as

monosaccharide composition, glycosidic linkages, backbone substitution and crosslinking play an important role in determining the fermentative behavior of gut microbiota (Botham et al., 1998). The effects of these characteristics are evident in the differences in fermentation rates and products.

In vitro fecal fermentation of cereal brans, which contain the insoluble dietary fiber, occurs to a lower extent and at a slower rate than for soluble dietary fibers (Bourquin et al., 1996; Karppinen et al., 2001; Kedia et al., 2009). Because of this, many attempts have been made to modify insoluble dietary fibers to facilitate their incorporation into foods without a detrimental effect to organoleptic properties and to improve their fermentability. The physicochemical properties of dietary fibers that are nutritionally relevant and strongly involved in colonic function are particle size, bulk effect, surface area, hydration, and rheological properties which determine their fermentation patterns (Guillon & Champ, 2000). Processing techniques are applied to modify those properties for improved fermentability.

1.2.3.1 Hydrothermal Treatment

Dietary fiber can be modified through hydrothermal treatment to change the ratio between insoluble and soluble fiber, total dietary fiber content and physicochemical properties, but the degree of modification depends largely on the source of the dietary fiber and treatment conditions (Elleuch et al., 2011). For example, treating sorghum bran with hot water (130°C for 20 min), concentrated and exposed hemicelluloses and cellulose; thus resulting in an enhanced release of pentosans (Corredor et al., 2007). Extrusion is a commonly used approach in the literature for solubilization of dietary fiber,

though may be of less practical value to the food industry, because it disrupts the covalent and non-covalent bonds in the polysaccharide and protein moieties which leads to smaller and more soluble fragments (Wang et al., 1993). In another study, 20% of the dietary fiber was soluble in extruded whole grain wheat versus 15% in the raw whole grain (Björck et al., 1984). Increases in soluble dietary fiber due to extrusion processing are explained to occur due to two possible reasons; by the transformation of some insoluble to soluble dietary fiber or by the formation of additional soluble dietary fiber by trans-glycosidation (Vasanthan et al., 2002). These authors suggest that it is important to consider the source of dietary fiber when attempting its modification through extrusion processing as changes in soluble, insoluble and total dietary fiber profiles are source and perhaps variety dependent. For instance, extrusion of bran fractions from durum wheat did not result in an increase in soluble dietary fiber, but it significantly increased its insoluble dietary fiber fraction (Esposito et al., 2005). Also, extreme extrusion conditions can result in excessive degradation of the insoluble fiber fraction that results in conversion of low molecular fragments into sugars. This can explain the overall decrease in total dietary fiber of some extruded samples in the study of Gajula et al. (2008). Ralet and coworkers (1990) showed that extrusion causes an increase in the solubility of glucuronoarabinoxylans that arise from the wheat kernel pericarp and arabinoxylans from aleurone and endosperm cell walls. This study also reported that extrusion processing caused a significant increase in water absorption capacity of wheat bran.

Autoclaving is another type of hydrothermal method commonly used in the treatment of insoluble dietary fibers. Autoclave treatment resulted in solubilization of hemicelluloses

and increased swelling of the polysaccharide polymers which increased their susceptibility to microbial degradation (Andersson et al., 2003; Guillon et al., 1992). However, autoclaving may not be as effective in solubilizing insoluble dietary fibers with high lignin content such as those from sorghum and pearl millet. Researchers in the biofuels area, who have extensively studied the pre-treatment of highly lignified biomass for ethanol production, describe other harsher processing techniques. Similar to autoclaving, liquid hot water is based on the application of heat under high-pressure conditions. Although, with liquid hot water, higher temperatures (~200 °C) can be reached while the water remains in the liquid state due to increased pressure (Pérez et al., 2008). Liquid hot water treatment has been used on materials such as corn stover, sugar cane bagasse, and tree biomass to effectively hydrolyze hemicellulose and disrupt lignocellulosic structures (Cara et al., 2007; Laser et al., 2002; Mosier et al., 2005). Another commonly used processing technique is microwave treatment, which consists of heating a solvent suspended sample with microwave radiation. Microwaves are uniformly absorbed by the suspension and cause vibration of the molecules that create heat through friction (Mandal et al., 2007). This processing technique has been used as a pretreatment to enhance enzymatic digestibility of lignocellulosic biomass in combination with alkali and acids (Hu & Wen, 2008; Intanakul et al., 2003; Zhu et al., 2006). Because microwave radiation results in the rupture of cell wall matrices, the technique has also been used for the extraction of phenolic compounds from a variety of materials (Gallo et al., 2010; Spigno & De Faveri, 2009), as well as hemicelluloses from agricultural by-products (Wang et al., 2007; Yoshida et al., 2010). Since these processing techniques are used on highly lignified biomass to enhance its susceptibility to enzymatic

hydrolysis, it is possible that their application on insoluble dietary fibers could result in enhanced fermentability. Thus, they constitute a promising approach for the modification of insoluble dietary fibers from alternative cereals to generate fermentable carbohydrates.

1.2.3.2 Enzymatic Hydrolysis

The use of enzymes for the modification or solubilization of dietary fiber in order to improve its nutritional properties and functionality is common. For example, the use endoxylanases, which are enzymes that can hydrolyze the xylan backbone of arabinoxylans, are frequently used in bread mixtures to improve dough handling, oven spring and loaf volume properties (Courtin & Delcour, 2002). In addition, xylanases can partially solubilize water-unextractable arabinoxylans that results in improved nutritional properties by increasing the amounts of soluble fiber (Andersson et al., 2003). The treatment of dietary fiber with enzymes can vary in various aspects such as type of enzyme, treatment conditions and dietary fiber source which will determine the end results. When enzymes from Trichoderma strains, with β-glucanase and xylanase activities, were used on fiber from durum wheat, a significant increase in soluble dietary fiber was obtained (Napolitano et al., 2006). The researchers found that the conversion of cereal insoluble dietary fiber into soluble dietary fiber corresponds to the release of hydroxycinnamic acid moieties linked to the polysaccharide chains. In addition to solubilization, treating fiber material that is rich in insoluble arabinoxylans with endoxylanases results in an increased ability to absorb water and swell (Gruppen et al., 1993) which can enhance its susceptibility to microbial degradation. Rouao and Moreau (1993) reported that the swelling power of water-insoluble pentosans was doubled and

viscosity increased as a result of enzymatic solubilization. This was attributed to the breakdown of internal bonds that allowed greater expansion of the fiber constituent. Addition of endoxylanases from *Bacillus subtillis,* which particularly target water-insoluble arabinoxylans, resulted in their partial solubilization (Brijs et al., 2004). However, in this same study, increased solubilization of water-insoluble arabinoxylans affected pasta quality. Nonetheless, the use of endoxylanases in pasta processing resulted in an increased level of soluble dietary fiber content that was not lost during cooking (Ingelbrecht et al., 2001).

1.3 Conclusions

The literature presented above highlights the importance of the consumption of dietary fibers in order to provide fermentable substrate for the gut microbiota that plays a crucial role in the health of the host. It is evident that structural characteristics of dietary fibers determine their physicochemical properties that, in turn, affect their fermentability. Hydrothermal or enzymatic treatment of cereal arabinoxylans can generate a wide array of fermentable carbohydrate structures that differ in degree of solubility, composition, and structure that can ferment in different regions of the gastrointestinal tract and have prebiotic effects throughout the colon. However, few studies are found where the dietary fiber modification is evaluated in terms of fermentability and, if found, tend to use raw, alkali-extracted, and/or enzymatically-solubilized hemicelluloses. Furthermore, the majority has used the more common cereal sources such as oat, rye, barley or wheat. Studies on fermentability and physiological properties of modified dietary fibers from other grains sources such as sorghum and pearl millet are scarce or not available. Other dietary fiber sources with potential prebiotic effects are dicotyledonous, pseudocereals

such as amaranth and quinoa. Important differences exist in the fermentability between monocotyledonous cereals and dicotyledonous plants. It has been previously shown that the differences in fermentation and sugar degradation patterns between the two types of plants are related to the differences in their cell wall composition and structure (van Laar et al., 2002). This fact alone justifies the need to investigate the potential fermentative properties of pseudocereals along with less common cereal sources. Identifying different carbohydrate substrates or creating a variety of substrate mixtures through the modifications of insoluble dietary fibers and evaluating their resulting fermentation profiles would likely lead us to a different approach of attempting to modulate gut microbiota via diet.

CHAPTER 2. QUANTIFICATION AND CHARACTERIZATION OF DIETARY FIBERS FROM ALTERNATIVE GRAINS

2.1 Abstract

Dietary fibers from four alternative grains; quinoa, amaranth, sorghum, and pearl millet, was analyzed for its insoluble and soluble fiber content, composition, and structure. Total dietary fiber content ranged between 11.4 to 9.3% for all four samples where amaranth and sorghum had the highest amounts. For quinoa and amaranth, 78.0% of its dietary fiber was insoluble and around 85.0% for sorghum and pearl millet. Insoluble dietary fiber (IDF) from quinoa and amaranth was mainly composed of galacturonic acid, arabinose, galactose, xylose and glucose. Linkage analysis indicated that IDF was composed of homogalacturonans and rhamnogalacturonan-I with arabinan side-chains (~55-60%), as well as highly branched xyloglucans (~30%) and cellulose. For both pseudocereals, 22% of total dietary fiber was soluble; a higher proportion than that found in sorghum and pearl millet (13.0% and 15.0%, respectively). The soluble fiber (SDF) was composed of glucose, galacturonic acid and arabinose; for amaranth, xylose was also a major constituent. Xyloglucans made up ~40-60% of the SDF and arabinose-rich pectic polysaccharides represented ~34-55%. The IDF fractions of sorghum and pearl millet were mainly composed of xylose, arabinose and glucose, indicating that arabinoxylans (AXs) were the predominant hemicellulose. Linkage analysis revealed

minor differences in the structure of the AXs found in the cereals. AXs from sorghum had a higher degree of branching than those from pearl millet, which had long stretched of un-substituted xylan. Around 80% of the IDFs from both cereals were constituted by AXs. The minor SDF fraction from the cereals was mainly composed of mannose and glucose, and glucomannan content was low. There were small amounts of highly branched AXs. Quinoa and amaranth grains had a comparable amount of dietary fiber as sorghum and pearl millet, the composition of their dietary fibers differed significantly. Since they are processed and consumed in the same ways as cereals, they prove to be good alternative grains for the supplementation of dietary fibers in the diets and/or the development of functional fibers.

2.2 Introduction

Evidence arising from epidemiological studies have linked low levels of dietary fiber in Westernized diets to diseases of the large bowel, increased risk for the onset of obesity, cardiovascular disease (Everson et al., 1992; Marlett & Vollendorf, 1993); and Type 2 diabetes (Champ et al., 2003; Kaline et al., 2007). Although a clear-cut relationship between dietary fiber and these diseases is difficult to establish because their etiology is often multifactorial, it is now widely recognized that dietary fiber plays an important role in a variety of physiological functions and can be used in the prevention and treatment of some diseases. Thus, consumers are becoming increasingly aware of the benefits of including a variety of cereal grains as a major portion of their diets. Increased consumption of cereals should spark consumer interest to seek out products made from alternative grains other than from common bread wheat cultivars. Consequently, food

scientists are now prompted to develop palatable food products with higher dietary fiber content and to search for alternative sources of dietary fibers that can promote health.

While cereal grains and their by-products are known to be good sources of dietary fiber, there is a newfound interest in alternative grains where less is known regarding the dietary fiber component. The pseudocereals quinoa (*Chenopodium quinoa* W.) and amaranth (*Amaranthus caudatus* L.), which are dicotyledonous species of Andean origin, have good nutritional profiles and are now considered as health-promoting foods. It has been reported that the content of lysine, methionine and cysteine is higher in protein from these pseudocereal grains than in most food proteins of plant origin (Bressani, 1989; Ruales & Nair, 1992) and that they are rich sources of iron, copper, manganese and zinc (Nascimento et al., 2014). Research on quinoa and amaranth has mainly focused on composition of the whole seed, protein quality, starch functionality, as well as incorporation into food products made with cereal flours. Some of the studies on whole seed composition have reported on its dietary fiber content; amaranth has a dietary fiber content that ranges from 8.0-16.0% of the whole seed (Berghofer & Schoenlechner, 2002) and 33.0-44.0% of it is soluble (Pedersen et al., 1990). Similar to amaranth, total dietary fiber content of quinoa (8.9%) is in the same range as found in cereal grains and leguminous seeds (Ranhotra et al., 1993).

Besides these pseudocereals, sorghum is more commonly used for animal feed in developed countries though has gained some popularity among food processers and consumers. Pearl millet is consumed as food grain in developing countries but has also gained some interest in developed countries due to its marketing opportunities as a

gluten-free grain. Much like the pseudocereals, research on the health effects sorghum and pearl millet is limited. Sorghum is known to be a slow digesting cereal and a recent study by Poquette et al. (2014) reported that incorporating whole sorghum grain into a food product can help to modulate glucose and insulin levels in healthy individuals. Sorghum flour has been incorporated into gluten-free baked goods and is easily processed by extrusion, micronization, and other commonly used processing techniques (Lemlioglu-Austin, 2014). Reports on the use of millets in food products are scarce, but their flours have good water and oil absorbing capacity, emulsion stability, and foaming capacity (Devisetti et al., 2014). Interest in sorghum and the millets has increased due to their significant phytochemical content, which may have health-benefitting properties (Taylor et al., 2014).

Interest in alternative grains has increased, and quinoa, amaranth, sorghum, and millet are viewed as good candidates for supplementation or substitution of common cereal grains. The composition and structure of dietary fibers from these cereals has been reported. However, a thorough analysis of composition and structural features has not been done for quinoa and amaranth dietary fibers and will be emphasized in this chapter. Knowledge of specific structural characteristics of dietary fibers from these alternative grains will help in understanding their potential as sources for functional fibers for colon health and associated whole body health conditions.

2.3 Materials and Methods

2.3.1 Cereal and Pseudocereal Grains

The alternative grains chosen for this study were two cereals, sorghum and pearl millet, and two pseudocereals, quinoa and amaranth. Sorghum and pearl millet grains were of African origin, purchased from Alif Group (Dakar, Senegal). Quinoa and amaranth grains were purchased from Shiloh Farms (New Holland, PA) and were certified organic products from Bolivia and Peru, respectively. For reference, wheat and maize were included in the study. Samples of wheat and maize grains were obtained from Nestlé Research Center (Lausanne, Switzerland) and Corn Products (Bedford Park, IL), respectively. Whole grains flours were prepared from all 6 grains by grinding dried grains in a Cyclotec 1093 Sample Mill (Foss North America, Eden Prairie, MN) to pass through a 0.8 mm screen. Whole grain flours were partially defatted with hexane (flour:hexane, 1:7 [w/v]) for 60 min in the case of flours from cereals and for 120 min in the case of flours from pseudocereals. Defatted whole grain flours were air-dried overnight.

2.3.2 Insoluble and Soluble Fiber Contents

The insoluble and soluble fractions of dietary fiber from the 6 grains were measured with a standard enzymatic-gravimetric method (AOAC Method 991.43). Briefly, 1 g of dried and defatted whole grain flour samples was subjected to a sequential enzymatic digestion with heat-stable α-amylase, protease and amyloglucosidase to remove starch and protein. The insoluble dietary fiber residue was collected by filtration and then washed, dried and weighed. The filtrates and water washings were pooled and precipitated with 95%

ethanol to collect soluble dietary fiber. Precipitates were filtered and dried. The insoluble and soluble dietary fibers collected were weighed and corrected for protein and ash content. Protein was measured by Dumas method (N x 6.25) and ash content was determined by incineration of sample at 525 °C for 5 hours. The values for each dietary fiber fraction are the average of triplicate measurements and total dietary fiber was calculated as the sum of insoluble and soluble dietary fibers.

2.3.3 Development of an Isolation Procedure for Insoluble and Soluble Dietary fiber Fractions

Large amounts of insoluble dietary fiber (IDF) samples from each alternative grain in their natural form were required for further analysis and experimentation. A procedure for the isolation of IDF and soluble dietary fiber (SDF) fractions was developed applying the basic principles of the standard enzymatic-gravimetric method used for the determination of dietary fiber content. This basic protocol was chosen to isolate each fiber fraction without significantly changing their physicochemical characteristics and it was modified to isolate IDF at a larger scale (Appendix 1). Briefly, 100-200 g of partially defatted whole grain flour was suspended in water (1:10, w/v) and heated to 90 °C. Heat-stable α-amylase (4 mL, Sigma A3403) was added to the suspension and incubated for 2 h with constant stirring. A second dose of α-amylase was added after the 2 h and incubation proceeded for 4 h with constant stirring for a total of 6 h. The suspension was filtered through vacuum-aided Buchner funnel with glass-fritted filter (Coarse, 40-60 μm). Filtrate was collected separately and residue was resuspended in water (1:10, w/v), stirred and cooled down to 50 °C. Protease (5 mL, Sigma P1236) was

added to the suspension and incubated for 4 hours with constant stirring. Whole grain flour slurry was filtered again and filtrates combined with previously collected filtrates. Amylase and protease incubations were repeated on IDF suspended in water (1:5, w/v). Incubation with amyloglucosidase (4 mL, Sigma A7095) was carried out on the washings containing SDF. After incubations with enzymes, insoluble dietary fiber was washed twice with water and 80% ethanol and dried in convention oven at 50 °C. Soluble fiber filtrates were dialyzed (MWCO 12-14 kDa) and freeze-dried.

2.3.4 Compositional Analysis of Dietary Fiber Samples

Moisture content of the samples was determined by loss in weight upon drying in a convection oven at 103 °C for 24 hours. The amount of residual starch in the isolated IDFs and SDFs was determined using an enzyme assay kit [Total Starch (AA/AMG Method), Megazyme, Wicklow, Ireland] and protein content was determined by the Dumas method (N x 6.25) at an external laboratory (Covance, Battle Creek, MI). Ash content was determined by loss in weight upon incineration in a muffle furnace at 525 °C for 5 hours. Lignin content of the insoluble dietary fiber samples was measured as described in the AACC International Official Method 32-25 (AACC, 2000) with a two-step 72% sulfuric acid hydrolysis of a 50 mg sample of IDF. First, samples were allowed to stand at 30 °C for 60 min with intermittent mixing to aid the acid dispersion throughout the sample. Then, the acid was diluted with water and samples were autoclaved at high-pressure setting for 60 min. The hydrolysate was filtered through No. 2 Pyrex fritted-glass filters while still warm. The residue collected in the filter was dried at 105 °C for 16 h and then incinerated for 1 hour at 525 °C. The loss in weight after

incineration was recorded and used to calculate the Klason lignin content of each sample. All the analyses described were performed in duplicate samples for each dietary fiber fraction for all 6 grains.

2.3.5 Monosaccharide and Glycosyl-linkage Composition of Dietary Fiber Samples

Neutral sugars in the dietary fiber samples from cereals were determined as alditol acetates prepared after 2 M TFA hydrolysis (60 min at 121 °C). Released monosaccharides were reduced and acetylated as described by York et al., (1986). In case of dietary fiber samples from pseudocereals, alditol acetates from constituent monosaccharides were prepared using the protocol described by Pettolino et al., (2012) that includes a carboxyl reduction to allow for the quantification of uronic acids. Alditol acetates in acetone were quantified by gas chromatography using a capillary column SP-2330 (SUPELCO, Bellefonte, PA) with the following conditions: injector volume, 2 µl; injector temperature, 240 ºC; detector temperature, 300 ºC; carrier gas (helium), velocity 1.9 meter/second; split ratio, 1:2; temperature program was 160 ºC for 6 min, then 4 ºC/min to 220 ºC for 4 min, then 3 ºC/min to 240 ºC for 5 min, and then 11 ºC/min to 255 ºC for 5 min.

Glycosyl-linkage composition of the dietary fiber samples from cereals was determined by the method described by Carpita and Shea (1989) with some modifications. Briefly, dietary fiber samples were dissolved or suspended (insoluble fibers) in anhydrous DMSO and methylated with n-Butyl Lithium (Sigma 230707). The methylated samples were hydrolyzed with 2 N TFA (60 min at 121 ºC). The hydrolyzed samples were dissolved in 1 M ammonium hydroxide and aldehyde groups were reduced with a DMSO solution

containing sodium borodeuteride (20 mg/ml). Glacial acetic acid was added drop wise to stop reaction and acetylation was done by addition of 1-methylimidazole and acetic anhydride. A different method, than the one used for structure analysis of cereal fibers was used to determine glycosyl-linkage composition of dietary fibers from pseudocereals. Partially methylated alditol acetates were prepared as described by Pettolino et al., (2012) which included a carboxyl reduction to allow for the determination of uronic acids linkages. Partially methylated alditol acetates in acetone were quantified by GC-FIDMS (7890A-5975C MSD, Agilent Technologies, Inc., Santa Clara, CA, USA) using a SP-2330 capillary column (injector volume, 1 µl; injector temperature, 240 ºC; detector temperature, 300 ºC; carrier gas, helium: 1.9 meter/second; split ratio, 100:1; temperature program, 100 ºC for 2 min, 8 ºC/min to 240 ºC for 20 min.

2.3.6 Data Analysis

Statistical analysis of the data was performed with SAS Software (version 9.3, SAS Institute, Cary, NC). Statistical differences, which were defined as $P < 0.05$, among insoluble and soluble dietary fiber contents and chemical components of each dietary fiber fraction (moisture, ash, protein, total starch and lignin) were analyzed using ANOVA in conjunction with Tukey's HSD test to determine significant differences among means. All data presented in tables is expressed as means ± standard error.

2.4 Results and Discussion

2.4.1 Determination of Dietary Fiber Contents in Alternative Grains

Cereals are good sources of digestible and indigestible carbohydrates (Nyman et al., 1984). Increased interest in dietary fiber consumption has led to the search of alternative

sources of dietary fiber that can be used for design and development of fiber enriched food products and/or fermentable carbohydrate substrates. Total dietary fiber contents (TDF) of defatted whole grain flours (WGF) from quinoa and amaranth were comparable to the TDF content of cereal defatted WGF (Table 2.1). These results confirmed previous reports that quinoa and amaranth have a dietary fiber content that is in the same range as found in cereal grains. TDF content of defatted WGF from maize was significantly lower than both of the pseudocereals. Amaranth defatted WGF had statistically similar contents of IDF and SDF compared to quinoa defatted WGF. Maize IDF content was lowest among all samples, though not significantly different from quinoa. IDF contents of 8-9% for samples of alternative grains did not differ significantly. In comparison to the reference grains used here, sorghum and amaranth were in the same grain grouping with the highest IDF content as wheat. On a flour weight basis, the pseudocereals still had generally high IDF levels that were comparable to cereals. On the other hand, around 22.0% of the TDF in quinoa and amaranth was soluble compared to 13.0-15.0% for the sorghum and pearl millet, respectively. Only wheat soluble dietary fiber content was not significantly different than quinoa. Other studies have reported SDF values for quinoa and amaranth that represent around 12.0-50.0% of their TDF content; which is wide range however, variations in genotype, growth conditions and method of analysis will result in different values for amounts of fiber components. Maize SDF content was significantly lower than all samples. Results showed that quinoa and amaranth provide a higher proportion of SDF than the cereals analyzed here. Although the pseudocereal grains investigated here are dicotyledonous species, they are consumed in the same ways as cereal grains. Previous studies have reported on their protein and mineral contents

showing that quinoa and amaranth have good nutritional profiles. Their proteins are higher in lysine, methionine and cysteine than other proteins of plant origin (Bressani, 1989; Ruales et al., 1992) and they are rich sources of iron, copper, manganese and zinc (Nascimento et al., 2014). Depending on the specific compositional and structural features of their dietary fibers and because many of the health-promoting properties of dietary fiber are attributed to the fermentability of SDF, the nutritional value of these pseudocereals may go beyond their rich mineral content and high quality protein.

2.4.2 Isolation of Insoluble and Soluble Dietary Fibers from Alternative Grains

A preparative-scale isolation procedure was developed to obtain sufficient amounts of dietary fiber samples for further analysis and experimentation. The standard protocol of enzymatic-gravimetric determination of dietary fiber was used as the basis for this procedure because it guaranteed the intact isolation of the non-starch polysaccharides contained in their dietary fibers. Other isolation methodologies that use acidic or basic solutions to obtain non-starch polysaccharides from plant and grain sources result in a modification of their physicochemical characteristics (Maes & Delcour, 2001; Weightman et al., 1994). Table A (Appendix 1) lists the details of the isolation procedure to obtain the IDF fraction from whole grain flours. The procedure, which included various extensive incubations with α-amylase and protease, proved to be successful for the isolation of both IDF and SDF fractions from each grain with low starch and protein contents. Due to initially high starch content of the whole grain flours, ranging from 44.0 to 56.0%, two incubations with α-amylase were required to remove the majority of starch. Two incubations with protease further aided in the removal of residual starch as the

protein that sometimes surrounds starch granules was hydrolyzed. Water and 80% ethanol washings of the fiber samples were important in order to eliminate free monosaccharides that remained after the enzymatic treatments. Water washings collected after isolation of IDF fractions were pooled and treated with amyloglucosidase. After enzyme treatment, they were mixed with ethanol to reach a 95% aqueous ethanol concentration. Mixtures were stirred for 30 min and allowed to stand at 4 °C overnight to precipitate soluble fibers. Both fiber fractions were collected, dried in convection oven at 50 °C for 24 hours and ground to a powder (particle size < 500 μm).

2.4.3 Chemical Composition of Isolated Insoluble and Soluble Dietary Fibers from Alternative Grains

The composition of the IDF fractions that were isolated from defatted WGF is presented in Table 2.2. Due to repeated amylolytic digestions in the isolation procedure, IDF fractions had low total starch content, ranging from 1.0% to 2.0% with the cereals being somewhat higher. Protein contents of the four IDF samples were also low and in a narrow range (1.0 – 2.1%).

The IDF fractions from both pseudocereals contained higher ash content than the cereals but only amaranth was significantly higher. These results are supported by previous reports showing that mineral contents of quinoa seeds are higher than those of cereals such as wheat (Koziol, 1992; Ranhotra et al., 1993). Lignin content was highest in sorghum and pearl millet (20.1 and 17.1%, respectively) and approximately 10% in both wheat and maize. Quinoa IDF was slightly less at ~9%, but lignin content for amaranth was significantly lower (~5%) than the rest. Lignin content for bran from whole quinoa

seeds has been reported to range from 6.0-7.0% (Repo-Carrasco-Valencia et al., 2009b), which is similar to what has been reported here for quinoa IDF. Since lignin, a high molecular weight polymer of aromatic compounds, serves to entrap the polymers that make up the cell wall, it renders the polymers insoluble and difficult to isolate or degrade (MacDougall & Selvendran, 2001). The lower lignin content found in the IDF fractions of the pseudocereals, especially for amaranth, likely contributes to their higher proportion of SDF content than cereals. Accordingly, lignin content correlated negatively (R^2 = -0.88) with the SDF content.

Similar to the IDF samples, SDF samples also had small amounts of starch and protein. Total starch contents for SDF were lower (<1.0%) for all six samples compared to IDF. In general, ash content (Table 2.3) was higher in SDF than in IDF for all samples; sorghum and quinoa had the highest values. This may be due to the association of minerals with endospermic tissue instead of the outer layers of the grain that are more lignified and insoluble. Most of the minerals in quinoa seeds are found in embryonic tissue as opposed to the pericarp (Konishi et al., 2004). Ash content in amaranth SDF was the lowest of all six samples but comparable to ash content found in its IDF fraction.

Total nonstarch polysaccharide (NSP) content in each IDF sample was estimated by difference and content ranged between 70.0-83.0%. Due to their higher lignin content, sorghum and pearl millet had total NSP contents near the lower end of that range and the opposite was the case for the pseudocereals. Total NSP content for SDF samples ranged from 79.0 to 90.0%.

2.4.4 Monosaccharide and Glycosyl-linkage Composition of Insoluble and Soluble Dietary Fibers from alternative Grains

2.4.4.1 Pseudocereals: Quinoa and Amaranth

Previous studies have reported on the monosaccharide composition of dietary fiber from quinoa and amaranth (Bunzel et al., 2005; Cordeiro et al., 2012; Pedersen et al., 1990). In this study it was found that IDFs from quinoa (QUI_{IDF}) and amaranth (AMA_{IDF}) were principally composed of galacturonic acid, arabinose, xylose, glucose, and galactose, presented in Table 2.4. Given that the starch content of these samples was low (1.3% QUI_{IDF}; 1.2% AMA_{IDF}), the bulk of the analyzed glucose was attributed to xyloglucans and cellulose, which are both characterized by a (1→4)-β-D-glucose backbone and xyloglucans are the major cross-linking hemicelluloses in the cell walls of all dicotyledonous seeds (Carpita et al., 2000; Selvendran, 1984). Interestingly, quinoa and amaranth seeds are consumed in the same way as cereals and, as previously mentioned, they have comparable dietary fiber contents, however, the monosaccharide composition of quinoa and amaranth indicates that these IDFs resemble that of tissues from fruits, vegetables, and leguminous seeds. An important difference between these pseudocereals and fruits and vegetables is that dietary fiber in pseudocereal seeds is dehydrated as opposed to the hydrated tissues from fruits and vegetables. Fruits and vegetables are usually consumed in a state, in which the tissues are characterized by high water content and low amounts of lignin (Eastwood, 1992), which helps explain their lower dietary fiber contents (Johnson & Southgate, 1994). The dietary fiber content of fruits and vegetables ranges from 1.5-2.5 g/100 g of dry weight (Marlett & Vollendorf, 1993;

Selvendran & Robertson, 1994). This is considerably lower than the sources of dietary fiber studied here which contained between 9-12 g/100 g of dry weight (Table 2.1). Although the monosaccharide compositions of dietary fibers from the pseudocereals and those from fruits, vegetables and leguminous seeds appear to be similar, the morphological differences between the types of tissues that constitute dietary fiber may be an indication that the structural features of the component polymers and the interactions among them will vary.

Compositional data of the IDF from quinoa and amaranth indicates that pectic polysaccharides are the dominant polymers in these fractions because galacturonic acid alone constitutes around 30% of total monosaccharide content. Linkage analysis of QUI_{IDF} and AMA_{IDF} provided further proof (Table 2.4). The majority of galacturonic acid was found to be (1→4)-linked that is characteristic of a galacturonan backbone. In addition, the rhamnosyl residues, which constituted ~7% of total monosaccharide content, were (1→2)-linked. Linkages for galacturonic acid and rhamnosyl residues found in QUI_{IDF} and AMA_{IDF} are typical of a pectic polysaccharide of the RG-I family. The relatively low content of rhamnose and the high proportion of (1→4)-linked-galacturonic acid indicate that the pectic polysaccharides in these IDFs are mainly composed of homogalacturonans interspersed with small stretches of RG-I. According to Mohnen (2008), homogalacturonans make up to 65.0% and RG-I represents 20.0-35.0% of the pectin found in plant cell walls. In addition, lower values of Rha/GalA ratio are indicative of a higher proportion of homogalacturonans (Coenen et al., 2007) and the Rha/GalA ratios for QUI_{IDF} and AMA_{IDF} were 0.22 and 0.23, respectively. The

Rha/GalA ratios for QUI_{IDF} and AMA_{IDF} were lower than those reported for apple (0.66), beet (0.35) and citrus (0.48) pectins (Thibault et al., 1993) and comparable to soy pectin (0.28) (Voragen et al., 2001). However, other reports of Rha/GalA ratios of the same fruit sources vary significantly. Therefore, a direct comparison between quinoa and amaranth, and other sources of pectic polysaccharides, is difficult to make because the ratio depends on a variety of factors. In this study, arabinose residues from QUI_{IDF} and AMA_{IDF} were found to be as terminal arabinofuranose (T→) and (1→3), (1→5)- and (1→3,5)-linked and galactose was found as terminal galactopyranose and (1→4)-linked. These linkage-types indicate that the RG-I sections of the pectic polysaccharides have arabinan and galactan side chains. It has been previously reported that most of the arabinose present in amaranth insoluble fiber was found to be (1→5)-linked (Bunzel et al., 2005). In addition, the results presented here are also in accordance with data presented by Cordeiro et al. (2012) that pectic polysaccharides from quinoa consisted of RG-I type polysaccharides with (1→5)-linked arabinan side chains substituted at O-3 and galactan side chains. Unlike the present study, other fiber constituents besides pectic substances were not reported.

As previously stated, xyloglucans are the major crosslinking hemicelluloses of dicot seeds; in general, they represent around 20.0-25.0% of the primary cell walls of dicotyledonous plants (Hayashi, 1989). Xyloglucans are structurally related to cellulose since they are commonly found non-covalently associated with it in plant cell walls (Hayashi et al., 1984). The main structural difference between cellulose and xyloglucan is that the latter is typically branched with single-unit α-D-Xyl*p* (**X**), dimeric β-D-Gal*p*-

(1→2)-α-D-Xyl*p* (**L**), α-L-Ara*f*-(1→2)- α-D-Xyl*p* (**S**), and oligomeric α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→2)-α-D-Xyl*p* (**F**), α-L-Ara*f*-(1→3)-α-L-Ara*f*-(1→2)-α-D-Xyl*p* (**T**) and α-L-Gal*p*-(1→2)-β-D-Gal*p*-(1→2)-α-D-Xyl*p* (**J**) side chains (Fry et al., 1993). Monosaccharide composition of the pseudocereal IDF fractions showed that QUI_{IDF} was composed of 9.9% xylose and 21.7% glucose, and AMA_{IDF} was composed of 12.7% xylose and 18.3% glucose (Table 2.4). Methylation analysis showed that the majority of the glucose found in QUI_{IDF} and AMA_{IDF} was (1→4)-linked, which is characteristic of a xyloglucan backbone, and a small proportion was found as (1→4,6)-linked glucose. In addition, xylosyl residues were found as (1→2)-linked-xylosyl. Therefore, the xylose and glucose contents in both of these IDF fractions were attributed to the presence of xyloglucans. These structural features suggest that the xyloglucans of QUI_{IDF} and AMA_{IDF} are mainly branched with disaccharide **L** side chains or oligomeric **F** and/or **J** side chains. The Xyl/Glc ratios for the xyloglucans found in QUI_{IDF} (Xyl/Glc = 0.46) and AMA_{IDF} (Xyl/Glc =0.69) furthermore indicate that they have a considerable degree of branching. Additionally, glucose from cellulose was quantified in the IDF samples by subjecting it to a harsh sulfuric acid hydrolysis that was preceded by 2 M TFA hydrolysis to eliminate the hemicelluloses. QUI_{IDF} and AMA_{IDF} contained 6.0% and 7.0% glucose from cellulose, respectively. However, these values may be underestimated since amorphous cellulose may be hydrolyzed by the 2 M TFA under the conditions used, therefore it is assumed that a portion of the total glucose arising from cellulose is lost during the first acid hydrolysis step.

In order to get an idea of the overall composition of these IDFs, the amounts of each polysaccharides were estimated based on the sum of the mol % values for the monosaccharides in their corresponding linkage-types. Xyloglucan content was estimated to be ~30% for both QUI_{IDF} and AMA_{IDF} based on the sum of the mol % values for (1→2)-linked xylose, (1→4)-linked and (1→4,6)-linked glucose and, (1→2,6)-linked-galactose and terminal-fucose; although residual starch and cellulose may result in an overestimation. In addition, pectic polysaccharides were estimated to constitute approximately 55% QUI_{IDF} and 59% AMA_{IDF} based on the sum of mol % values for terminal and (1→4)-linked-galacturonic acid; terminal and (1→5)-linked-arabinose; (1→4)-linked-galactose, and terminal- and (1→2)-linked-rhamnose.

The SDF fractions of both pseudocereals were mainly composed by galacturonic acid, galactose and arabinose, which are indicative of the presence of pectic polysaccharides (Table 2.5). Homogalacturonans, indicated by the significant amounts of galacturonic acid and lack of rhamnosyl residues, are the important constituent of the pectic polysaccharides. The low xylose content and high amount of galacturonic acid found in QUI_{SDF} suggests that this fiber sample is mainly composed of pectic polysaccharides, namely, homogalacturonan and arabinan. Based on the sum of terminal, (1→3)-, (1→3,5)- and (1→5)-linked arabinose plus the mol % value for galacturonic acid, pectic polysaccharides were estimated to constitute ~55% QUI_{SDF} and 34% AMA_{SDF} of the SDF fractions. As in the case of estimated xyloglucan content, the exact amount of pectic polysaccharides cannot be determined here. Although not as significant as the other monosaccharides, mannose was found in the SDF fractions in considerable amounts.

Galactomannan content was measured in pseudocereal SDF fractions and both contained low amounts (0.5% QUI_{SDF}; 0.3% AMA_{SDF}).

Other main monosaccharide components of QUI_{SDF} and AMA_{SDF} were glucose, galacturonic acid and arabinose, in the case of AMA_{SDF}, xylose was also present in a significant amount. Owing to the low starch content in these samples, the glucose found would be derived, in the majority, from xyloglucans; however, xylose was present in a low amount in quinoa. According to the monosaccharide composition of these SDF samples, the glucan polymer from quinoa appeared to have a much lower degree of branching (Xyl/Glc = 0.11 QUI_{SDF}) than its IDF fraction, that was not the case for amaranth (Xyl/Glc = 0.71 AMA_{SDF}). As evidenced by the results of methylation analysis, a portion of QU_{SDF} xyloglucan, which has slightly higher galactose content than AMA_{SDF}, was substituted with a small proportion of **F** side chains where glucose was substituted at C-6 with a trisaccharide composed of xylose, galactose and fucose residues. In the case of AM_{SDF}, the xyloglucans had a higher degree of branching as all xylose was present in a (1→2)-linkage. The data indicates that the xyloglucans have two main types of branches; **L** side chains composed by disaccharides of xylose and galactose, and **F** side chains. Xyloglucan amount in the SDF fractions was estimated to comprise 40-60% QUI_{SDF} and 60-70% AMA_{SDF} based on the sum of (1→2)-linked-xylose; terminal (1→4)- and (1→4,6)-linked-glucose, and (1→2,6)-linked-galactose. Xyloglucan side chains may also contain arabinose, however arabinose was not taken into account to estimate total amounts of xyloglucans because it is a component of pectic polysaccharides as well. Since these pseudocereals have not been previously studied for their complete fiber

characteristics, the exact composition of the xyloglucan side chains is not known. Thus, the estimated amounts of xyloglucans may be underestimated here.

2.4.4.2 Cereals: Sorghum and Pearl Millet

On the other hand, dietary fiber from the bran of cereal grains, which constitutes the insoluble dietary fiber fraction, is mainly composed of acidic arabinoxylans that have varying degrees of branching and are cross-linked via ferulic acid esters to form complexes with other arabinoxylans or with cellulose and lignin (Izydorczyk& Biliaderis, 1995). The main monosaccharide constituents of sorghum IDF (SOR_{IDF}) are xylose and arabinose indicating that an arabinoxylan polymer is dominant in this fiber fraction (Table 2.6). Glucose is a lesser, though still significant, component in this sample, which may correspond to minor amounts of residual starch (1.0% of SOR_{IDF}) and β-glucan (0.45% of SOR_{IDF}), but is likely mostly from cellulose that is released during acid hydrolysis. Cellulose content of sorghum bran was reported to be 11% of dry weight (Corredor et al., 2007) showing that cellulose is an important component of the insoluble fraction. In this study, cellulose content in SOR_{IDF} and PMI_{IDF} is estimated to be around 12-15%, respectively. In addition, galactose and glucuronic acid are known to be constituents of side chains attached to the xylan backbone of arabinoxylans. Arabinoxylans substituted with glucuronic acid are abundant in sorghum and referred to as glucuronoarabinoxylans (Verbruggen et al., 1995; Verbruggen et al., 1993). Mannose, galacturonic acid, rhamnose and fucose are also present in small amounts.

The monosaccharide composition of pearl millet insoluble dietary fiber (PMI_{IDF}) was similar to that of SOR_{IDF}, as was expected. However, glucose content of PMI_{IDF} was half

the content of glucose in SOR_{IDF}. Glucose in PMI_{IDF} likely corresponds to minor amounts of residual starch (1.2% of PMI_{IDF}), β-glucan (0.18% PMI_{IDF}) of and cellulose. Another difference in monosaccharide composition between these two fiber samples is the higher galactose content and lower glucuronic acid content in PMI_{IDF} compared to SOR_{IDF}. This suggests that the branches on the xylan backbone of arabinoxylans from PMI_{IDF} have more galactose and single-unit glucuronic acid branches are present in lower amounts (Table 2.6). The glycosyl-linkage composition of PMI_{IDF} shows some differences in structural features compared to SOR_{IDF}. The mol % of (1→4)-Xylp in PMI_{IDF} accounts for the majority of the xylose linkages indicating that it has a significantly higher proportion of unsubstituted xylan backbone than SOR_{IDF}. In addition, linkage data for PMI_{IDF} shows an 8.3 mol % of di-substitution on the xylan backbone, which was not observed for SOR_{IDF}. Both terminal-xylose and terminal-arabinose were higher in PMI_{IDF} suggesting higher xylose content on the branches attached to the xylan backbone.

The monosaccharide composition of soluble dietary fiber from sorghum and pearl millet (SOR_{SDF} and PMI_{SDF}) is presented in Table 2.7. Mole percentages of glucose and mannose makeup 81.0% and 77.0% for SOR_{SDF} and PMI_{SDF} monosaccharide compositions, respectively, indicating that the samples mainly consist of glucomannan and β-glucan polymers. However, glucomannan and β-glucan contents were unexpectedly low. Glucomannan content for SOR_{SDF} was 0.64% and the β-glucan content was 0.44%. In the case of PMI_{SDF}, glucomannan content is higher, 0.72%, and the β-glucan content is lower, 0.27%, than SOR_{SDF}. Structural studies of the cell walls in monocot plants have shown that mixed-linkage β-glucans and glucomannans are cross-

linking glycans that tightly coat the cellulose microfibrils (Carpita et al., 2001) and are present in small amounts (Rao & Muralikrishna, 2004). Xylose is also present in a noticeable amount, which leads to the estimation of the presence of arabinoxylans as well. Uronic acids were also an important component of these soluble fiber samples. Glucuronic acid tends to be found as substituent moieties of arabinoxylans and galacturonic acid is part of pectins that are embedded between cells (Carpita & Shea, 1989). PMI_{SDF} had a higher uronic acid content (~ 9.0%) than SOR_{SDF} (~ 6.0%). It is important to consider that the amounts of these monosaccharides, obtained by acid hydrolysis of the constituent polymers, only provide an idea of which polysaccharides make up the sample, because monomers like galactose, glucose and arabinose are constituents of more the one polysaccharide type.

Analysis of the glycosidic-linkage composition of SOR_{SDF} and PMI_{SDF} revealed that the main structural differences between the samples are found in variations in the mannan polymer structure (Table 2.7). In the case of SOR_{SDF}, the majority of mannose was found in a (1→4)-linkage with a single substitution at position C-2 or C-6. Studies on structural determination of glucomannans have reported the acetylation of the mannan backbone, which typically ranges from 5-10% (Alonso-Saude et al., 2009), at position C-2 and/or C-3 (Hannuksela & Hervé du Penhoat, 2004). In addition to acetyl groups, glucomannans can also substituted at C-6 with galactose (Puls & Schuseil, 1993). On the other hand, mannose in PMI_{SDF} was also found in (1→4)-linkages mono-substituted at C-2 or C-6, but it also had comparable amounts of (1→6)-linked and un-substituted linear

mannan. In comparison, glucomannan from SOR_{SDF} was more branched than glucomannan from PMI_{SDF}.

2.4.4.3 Cereals: Wheat and Maize

The monosaccharide composition of IDFs from wheat (WHE_{IDF}) and maize (MAI_{IDF}) are comparable except for the galactose content, which was higher for MAI_{IDF} (Table 2.8). Similar to the other sorghum and pearl millet, arabinoxylans were the predominant hemicellulose. Arabinoxylans from WHE_{IDF} and MAI_{IDF} had a lower arabinose and higher xylose content compared to SOR_{IDF} and PMI_{IDF}. Glucose is also a significant monomeric unit in these fiber samples and can be attributed mainly to cellulose, but also starch and β-glucans. Starch (2.0% of WHE_{IDF} and 1.9% of MAI_{IDF}) and β-glucans (0.28% of WHE_{IDF} and 0.35% of $MAI_{IDF,}$ respectively) comprise about 20% of the total glucose. Cellulose has been reported to range from 30.0-35.0% in wheat bran unextractable cell wall material that was enzymatically destarched and proteolyzed (Maes & Delcour, 2002). Methylation analysis of WHE_{IDF} and MAI_{IDF} show differences in structural features of their arabinoxylans. WHE_{IDF} had a higher proportion of un-substituted xylan backbone compared to MAI_{IDF} whose xylan backbone was mainly mono-substituted at position C-3. MAI_{IDF} also has a higher terminal-xylopyranose and terminal-arabinofuranose suggesting a higher amount of xylose in the branches of MAI_{IDF} arabinoxylans and greater proportion of single-arabinofuranosyl side chains along the xylan backbone.

The monosaccharide composition of SDF from wheat and maize (WHE_{SDF} and MAI_{SDF}), presented in Table 2.9, differed between each other. For the most part, WHE_{SDF} is composed of mannose, xylose, glucose and arabinose indicating that arabinoxylans and

glucomannans (0.37%) are the predominant polymers in this sample. However, a portion of glucose can also be attributed to β-glucan (0.37% of WHE_{SDF}). On the other hand, MAI_{SDF} had mannose and glucose as its main monomer units which indicates that glucomannan was the predominant polymer athend the arabinoxylans constituted only a minor amount of the total non-starch polysaccharides. However, glucomannan content in MAI_{SDF}, expected to be found in high amounts, was low (0.65% of MAI_{SDF}). Also, β-glucan content in MAI_{SDF} was minor (0.29 g/100g of MAI_{SDF}), and lower amount than WHE_{SDF}. According to the results for linkage analysis, the mannan polymer from WHE_{SDF} is, in its majority, present in a (1→4)-linkage mono-substituted at position C-6. Similar to SOR_{SDF} and PMI_{SDF}, the mannan polymer from MAI_{SDF} was mono-substituted at C-2 or C-6. Linkage analysis for WHE_{SDF} also showed that the xylan backbone was mainly di-substituted with single xylopyranose or monomeric and oligomeric arabinose side chains. The arabinofuranosyl units attached to the xylan backbone had another constituent attached to it at position C-5. As well as the other SDF samples discussed previously, WHE_{SDF} and MAI_{SDF} also showed a high mole percentage of terminal glucose units.

2.5 Conclusions

IDF contents did not differ between pseudocereals and cereals, however pseudocereals had a greater amount of SDF than cereals. Protein and residual starch contents of the isolated IDF and SDF fractions were generally low for all dietary fiber samples indicating that starch and protein were efficiently removed during the large-scale isolation procedure. In general, ash content was higher in SDF fractions than in IDFs, which

suggests that more minerals in the whole grain flour bind to the soluble non-starch polysaccharides or are readily soluble. On the other hand, lignin content was highest for sorghum and pearl millet IDFs. Amaranth, which had a comparable IDF content to wheat, had the lowest lignin content.

Analysis of the monosaccharide and glycosidic-linkage composition of the IDF from pseudocereal samples showed that a rhamnogalacturonan with arabinan and galactan side chains along with xyloglucans were the main hemicelluloses. IDF from cereals was mainly composed of arabinose and xylose indicating that these fiber samples were predominantly made up of arabinoxylan polymers. Linkage analysis from cereals showed a few structural differences between sorghum and pearl millet. Arabinoxylans from sorghum insoluble fiber (SOR_{IDF}) were mostly mono-substituted with monomeric arabinofuranosyl side chains at C-3 positions of xylose units in the backbone. Insoluble dietary fiber from pearl millet (PMI_{IDF}) had arabinoxylans with a higher proportion of un-substituted xylan backbone and a noticeable amount of di-substituted backbone, which was not observed for SOR_{IDF}. In the case of SDF from maize, sorghum, and pearl millet, and, glucose and mannose were the main monomeric components. This composition was attributed to residual starch, β-glucan and glucomannans. In the case of soluble dietary fiber from wheat (WHE_{SDF}), xylose and arabinose were important monomeric constituents as well. This was attributed to the presence of arabinoxylans in addition to the previously mentioned polysaccharides. Pseudocereal SDFs were mainly composed of galacturonic acid, glucose, galactose, and arabinose. Thus, in addition to residual starch and xyloglucans, pseudocereal SDFs also contained a galacturonans with arabinan and

galactan chains.

Overall, the quantification and characterization of dietary fibers from alternative grains revealed that pseudocereals are good sources of TDF, comparable to cereals, and that they provided a higher proportion of SDF. The composition of their dietary fibers differed. Quinoa and amaranth have dietary fibers that are rich pectic polysaccharides and xyloglucans. Since this fiber composition is different than that of cereal fibers, the potential physiological and functional properties of quinoa and amaranth fibers should be investigated.

Table 2.1 Insoluble, soluble, and total dietary fiber contents of alternative grains

Dietary Fiber Fraction	% From Defatted Whole Grain Flour (dry basis)					
	Amaranth	Quinoa	Pearl Millet	Sorghum	Wheat	Maize
Insoluble	$8.9^{ab} \pm 0.3$	$7.7^{bc} \pm 0.6$	$7.9^{b} \pm 0.8$	$8.9^{ab} \pm 0.3$	$9.8^{a} \pm 0.9$	$6.5^{c} \pm 0.4$
Soluble	$2.5^{a} \pm 0.1$	$2.3^{ab} \pm 0.3$	$1.4^{c} \pm 0.2$	$1.4^{c} \pm 0.4$	$1.7^{bc} \pm 0.2$	$1.2^{c} \pm 0.1$
Total[a]	11.4	9.9	9.3	10.3	11.5	7.7

[a]Total dietary fiber = the sum of insoluble and soluble dietary fibers.
Values are the average of triplicate measurements.
Different letters within each row indicate significant differences ($P<0.05$)

Table 2.2 Composition of insoluble dietary fiber fractions from cereal and pseudocereals.

Constituent	% From Defatted Whole Grain Flour (dry basis)					
	Amaranth	Quinoa	Pearl Millet	Sorghum	Wheat	Maize
Moisture	6.3 ± 0.5	6.1 ± 0.1	6.1 ± 0.1	5.5 ± 0.1	6.2 ± 0.2	6.7 ± 0.1
Ash	2.8 ± 0.5	1.6 ± 0.2	0.6 ± 0.4	1.1 ± 0.1	0.7 ± 0.1	0.8 ± 0.1
Protein	2.1 ± 0.1	1.4 ± 0.3	2.2 ± 0.1	1.9 ± 0.2	1.1 ± 0.2	0.9 ± 0.3
Lignin	4.9 ± 0.3	8.6 ± 0.5	17.1 ± 0.9	20.1 ± 1.0	9.6 ± 0.7	9.9 ± 0.5
Starch	1.2 ± 0	1.3 ± 0	1.2 ± 0.1	1.0 ± 0.1	2.0 ± 0.1	1.9 ± 0
Nonstarch polysaccharides[b]	83	81	73	70	80	80

[a]Values are the average of duplicate measurements ± standard deviation
[b]Values for Nonstarch polysaccharides content were estimated by difference [100-(Moisture % + Ash % + Protein % + Lignin % + Starch %) = NSPs]

Table 2.3 Composition of soluble dietary fiber fractions from cereal and pseudocereals

Constituent	% From Defatted Whole Grain Flour (dry basis)					
	Amaranth	Quinoa	Pearl Millet	Sorghum	Wheat	Maize
Moisture	5.3 ± 0.7	9.3 ± 0.8	8.6 ± 0.8	9.0 ± 0.1	5.7 ± 0.4	9.0 ± 0.1
Ash	3.0 ± 0.3	9.4 ± 0.8	6.4 ± 0.1	10.5 ± 0.9	7.5 ± 0.7	6.0 ± 0.1
Protein	0.9 ± 0.1	0.9 ± 0.1	1.0 ± 0.2	0.9 ± 0.1	0.9 ± 0.1	1.4 ± 0
Starch	0.5 ± 0	0.8 ± 0	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.4 ± 0
Nonstarch polysaccharides[b]	90	80	84	79	85	83

[a]Values are the average of duplicate measurements ± standard deviation
[b]Values for Nonstarch polysaccharides content were estimated by difference [100-(Moisture % + Ash % + Protein % + Starch %) = NSPs]

Table 2.4 Monosaccharide and glycosyl-linkage composition (mol %) of insoluble dietary fiber fraction from quinoa and amaranth

Monosaccharides	QUI_{IDF}	AMA_{IDF}	Linkage[a,b,c]	QUI_{IDF}	AMA_{IDF}
Galacturonic Acid	28.5 ± 0.6	33.1 ± 0.2	[d]T-GalA →	5.2	5.3
			1 → 4	23.3	27.7
Arabinose	15.3 ± 0.1	13.8 ± 0.3	T-Ara →	10.5	8.1
			1 → 5	4.8	5.7
Xylose	9.9 ± 0.1	12.7 ± 0.2	1 → 2	9.9	12.7
Glucose	21.7 ± 0.4	18.3 ± 0.4	T-Glc →	1.1	1.1
			1 → 4	19.2	16.0
			1→ 4,6	1.4	1.2
Galactose	6.4 ± 0.0	7.9 ± 0.5	1 → 2,6	2.0	3.3
			1→ 4	4.3	4.7
Rhamnose	6.5 ± 0.0	7.4 ± 0.2	T-Rha →	0.4	0.9
			1 → 2	5.4	5.4
			1 → 2,4	0.7	1.1
Fucose	2.3 ± 0.0	2.6 ± 0.3	T-Fuc →	2.3	2.6
Mannose	3.2 ± 0.2	2.4 ± 0.2			
Glucuronic Acid	0.8 ± 0.2	1.7 ± 0.3			
Rha/GalA ratio	0.23	0.22			
Xyl/Glc ratio	0.46	0.69			

[a]Monosaccharide and glycosyl-linkage amounts are expressed as average mol % ± standard deviation

[b]Values are expressed as a proportion of all partially methylated alditol acetates present and the average of triplicate measurements

[c]The numbers in each linkage refer to the carbon on the monosaccharide involved in the bond (e.g. 1→ 4 indicates that carbon #1 of the first monosaccharide is linked to carbon #4 on the next monosaccharide)

[d]T→ refers to a terminal monosaccharide (the monosaccharide is only linked to another monosaccharide or polysaccharide constituent on one of its carbon molecules.

Table 2.5 Monosaccharides and glycosyl-linkage composition (mol%) of soluble dietary fiber fraction from quinoa and amaranth

Monosaccharides	**QUI_{SDF}**	**AMA_{SDF}**	**Linkage[a,b,c]**	**QUI_{SDF}**	**AMA_{SDF}**
Glucose	24.5 ± 0.4	31.2 ± 2.0	[d]T-Glc →	5.5	1.1
			1 → 4	14.6	27.8
			1 → 4,6	4.4	1.9
Galacturonic Acid	38.6 ± 0.3	18.7 ± 1.8	1 → 4	38.6	18.7
Arabinose	15.6 ± 0.1	11.5 ± 2.1	T-Ara →	--	2.61
			1 → 3	--	6.41
			1 → 3,5	8.51	0.93
			1 → 5	7.12	--
Galactose	7.3 ± 0.2	5.7 ± 0.3	T-Gal →	7.34	2.2
			1 → 2,6	--	3.6
Mannose	6.9 ± 0.2	5.4 ± 0.6			
Xylose	2.7 ± 0.3	22 ± 1.4	1 → 2	2.7	22
Xyl/Glc ratio	0.11	0.71			

[a]Monosaccharide and glycosyl-linkage amounts are expressed as average mol % ± standard deviation

[b]Values are expressed as a proportion of all partially methylated alditol acetates present and the average of triplicate measurements

[c]The numbers in each linkage refer to the carbon on the monosaccharide involved in the bond (e.g. 1→ 4 indicates that carbon #1 of the first monosaccharide is linked to carbon #4 on the next monosaccharide)

[d]T→ refers to a terminal monosaccharide (the monosaccharide is only linked to another monosaccharide or polysaccharide constituent on one of its carbon molecules.

Table 2.6 Monosaccharide and glycosyl-linkage composition (mol%) of insoluble dietary fiber fraction from sorghum and pearl millet.

Monosaccharides	SOR_{IDF}	PMI_{IDF}	Linkage[a,b,c]	SOR_{IDF}	PMI_{IDF}
Xylose	37.0 ± 0.3	43.1 ± 0.1	[d]T-Xyl →	10.8	12.3
			1 → 4	5.7	24.0
			1 → 3,4	21.7	--
			1 → 2,4	--	6.8
Arabinose	31.6 ± 0.1	34.7 ± 0.1	T-Ara →	14.6	20.4
			1 → 2,3	3.3	12.8
			1 → 2,5	5.3	--
			1 → 3,5	9.2	--
			1 → 5	--	1.1
Glucose	15.7 ± 0.2	7.6 ± 0.2	T-Glc →	1.2	1.7
			1 → 4	16.1	5.8
Galactose	3.9 ± 0.1	6.1 ± 0			
Mannose	2.7 ± 0.3	1.8 ± 0.2			
Galacturonic Acid	2.6 ± 0.3	1.8 ± 0.1			
Glucuronic Acid	2.5 ± 0.2	1.5 ± 0			
Fucose	2.4 ± 0.1	2.7 ± 0.3			
Rhamnose	1.5 ± 0	0.8 ± 0			
Ara/Xyl ratio	0.85	0.81			

[a]Monosaccharide and glycosyl-linkage amounts are expressed as average mol % ± standard deviation

[b]Values are expressed as a proportion of all partially methylated alditol acetates present and the average of triplicate measurements

[c]The numbers in each linkage refer to the carbon on the monosaccharide involved in the bond (e.g. 1→ 4 indicates that carbon #1 of the first monosaccharide is linked to carbon #4 on the next monosaccharide)

[d]T→ refers to a terminal monosaccharide (the monosaccharide is only linked to another monosaccharide or polysaccharide constituent on one of its carbon molecules.

Table 2.7 Monosaccharide and glycosyl-linkage composition (mol %) of soluble dietary fiber fraction from sorghum and pearl millet

Monosaccharides	SOR_{SDF}	PMI_{SDF}	Linkage[a,b,c]	SOR_{SDF}	PMI_{SDF}
Mannose	59 ± 0.2	54.3 ± 1.0	1 → 4	8.5	12.2
			1 → 6	10.4	13.1
			1 → 2,4	23.8	13.7
			1 → 4,6	16.3	15.4
Glucose	22.8 ± 0.2	23.3 ± 0.2	[d]T-Glc →	20.3	22
			1 → 4	2.4	1.4
Galacturonic Acid	5.1 ± 0.4	7.7 ± 0.1			
Galactose	4.2 ± 0.2	4.5 ± 0.3	1 → 2	4.2	4.5
Arabinose	3.0 ± 0	4.0 ± 0.2	T-Ara →	2.2	3.0
			1 → 5	0.9	1.0
Xylose	2.9 ± 0	3.1 ± 0	T-Xyl →	2.9	3.1
Rhamnose	1.7 ± 0.1	1.2 ± 0.1			
Glucuronic Acid	0.8 ± 0.2	1.2 ± 0.1			
Fucose	0.7 ± 0	0.6 ± 0.1			
Ara/Xyl ratio	1.03	1.3			

[a]Monosaccharide and glycosyl-linkage amounts are expressed as average mol % ± standard deviation

[b]Values are expressed as a proportion of all partially methylated alditol acetates present and the average of triplicate measurements

[c]The numbers in each linkage refer to the carbon on the monosaccharide involved in the bond (e.g. 1→ 4 indicates that carbon #1 of the first monosaccharide is linked to carbon #4 on the next monosaccharide)

[d]T→ refers to a terminal monosaccharide (the monosaccharide is only linked to another monosaccharide or polysaccharide constituent on one of its carbon molecules.

Table 2.8 Monosaccharide and glycosyl-linkage composition (mol %) of insoluble dietary fiber fraction from wheat and maize.

Monosaccharides	WHE_{IDF}	MAI_{IDF}	Linkage[a,b,c]	WHE_{IDF}	MAI_{IDF}
Xylose	47.5 ± 0.7	45.4 ± 0.1	[d]T-Xyl →	4.2	12.3
			1 → 4	34.8	13.9
			1 → 3,4	8.5	22.3
Arabinose	29.2 ± 0	24.6 ± 0.3	T-Ara →	15.2	19.6
			1 → 2	0.8	1.4
			1 → 3	1.8	--
			1 → 5	1.8	4.7
			1 → 2,3,5	9.6	--
Glucose	11.2 ± 0.1	11.6 ± 0.2	T-Glc →	0.5	0.9
			1 → 4	10.6	9.3
			1 → 4,6	--	1.9
Galactose	2.4 ± 0.1	6.8 ± 0.1	T-Gal →	0.5	3.4
			1 → 4	1.9	4.0
Mannose	3.0 ± 0.2	3.0 ± 0.1			
Galacturonic Acid	3.0 ± 0.4	3.7 ± 0.2			
Glucuronic Acid	1.2 ± 0.1	1.2 ± 0.1			
Fucose	2.2 ± 0.1	2.3 ± 0.1			
Rhamnose	0.4 ± 0	1.0 ± 0			
Ara/Xyl ratio	0.61	0.54			

[a]Monosaccharide and glycosyl-linkage amounts are expressed as average mol % ± standard deviation

[b]Values are expressed as a proportion of all partially methylated alditol acetates present and are the average of triplicate measurements

[c]The numbers in each linkage refer to the carbon on the monosaccharide involved in the bond (e.g. 1→ 4 indicates that carbon #1 of the first monosaccharide is linked to carbon #4 on the next monosaccharide)

[d]T- → refers to a terminal monosaccharide (the monosaccharide is only linked to another monosaccharide or polysaccharide constituent on one of its carbon molecules.

Table 2.9 Monosaccharide and glycosyl-linkage composition (mol %) of soluble dietary fiber fraction from wheat and maize.

Monosaccharides	WHE_{SDF}	MAI_{SDF}	Linkage[a,b,c]	WHE_{SDF}	MAI_{SDF}
Mannose	35.3 ± 0.6	56.3 ± 1.8	1 → 4	24.6	13.3
			1 → 6	10.6	15.9
			1 → 2,4	--	27.1
Xylose	27.9 ± 0.3	3.3 ± 0.2	[d]T-Xyl →	7.1	3.3
			1→2,3,4	20.8	--
Glucose	15.8 ± 0.1	27.4 ± 0	T-Glc →	14.7	27.4
			1 → 4	1.1	--
Arabinose	11.3 ± 0.4	3.1 ± 0.2	T-Ara →	3.4	1.3
			1 → 5	7.8	0.3
			1 → 2	--	1.2
Galacturonic Acid	3.8 ± 0	4.2 ± 1.5	T-GalA →	2.9	3.1
Galactose	3.5 ± 0.6	3.3 ± 0.1	1 → 2	3.5	--
Fucose	1.6 ± 0.1	0.6 ± 0.1			
Rhamnose	0.5 ± 0	1.2 ± 0.1			
Glucuronic Acid	0.4 ± 0	0.7 ± 0.1			
Ara/Xyl ratio	0.41	0.94			

[a]Monosaccharide and glycosyl-linkage amounts are expressed as average mol % ± standard deviation

[b]Values are expressed as a proportion of all partially methylated alditol acetates present and are the average of triplicate measurements.

[c]The numbers in each linkage refer to the carbon on the monosaccharide involved in the bond (e.g. 1→ 4 indicates that carbon #1 of the first monosaccharide is linked to carbon #4 on the next monosaccharide)

[d]T- → refers to a terminal monosaccharide (the monosaccharide is only linked to another monosaccharide or polysaccharide constituent on one of its carbon molecules.

CHAPTER 3. SOLUBILIZATION OF INSOLUBLE DIETARY FIBERS BY HYDROTHERMAL TREATMENTS AND ENZYMATIC HYDROLYSIS

3.1 Abstract

The majority of dietary fiber from cereal and pseudocereal grains is insoluble. Thus, it is poorly fermentable and less likely to have a beneficial effect on the colonic microbiota. Enzymatically-isolated insoluble dietary fiber (IDF) from pearl millet, wheat, amaranth and quinoa was subjected to hydrothermal treatments and enzymatic hydrolysis to enhance and effect solubilization. The development of an effective non-chemical solubilization methods consisted of subjecting IDF suspensions (10% w/v, in water) to microwave radiation (MR), liquid hot water (LHW), and autoclave (AUT) treatments. Each treatment lasted 30 min and was controlled to reach a maximum temperature of 120 °C. Freeze-dried treated-IDF samples (1% suspensions, w/v) in water and enzymatically-hydrolyzed to further solubilize the IDFs. The enzyme treatment consisted of neutral protease (0.1 U/g IDF) for 4 h at 50 °C followed by feruloyl esterase (30 U/g IDF) for 4 h at 50 °C. Cereal treated-IDFs suspensions were subsequently treated with endoxylanase and cellulase (70 U/g IDF ea.) for 24 h at 50 °C. Pseudocereal IDFs were treated with endopolygalacturonase, and cellulase (70 U/g IDF ea.) for 24 h at 50 °C. Additional trials in the development of the enzymatic solubilization procedure included an enzymatic hydrolysis without protease incubation and another enzymatic hydrolysis

with endo-β-galactanase (100 U/g IDF) for the pseudocereal IDFs. Solubilized carbohydrates were quantified by the phenol-sulfuric acid method. In general, LHW and MR treatments solubilized comparable amounts of fiber and were more effective than AUT pretreatment. For cereals, LHW and MR treatments in combination with enzymatic hydrolysis resulted in the highest increase of soluble fiber content. Wheat and pearl millet IDF samples had 21.0% and 14.0% increase in soluble fiber, respectively. In the case of pseudocereals, MR+enzyme treatment resulted in higher solubilization for quinoa IDF (47.0% increase in soluble fiber) and LHW+enzyme treatment effected a higher solubilization in the case of amaranth IDF (54% increase in soluble fiber). Treatment of the IDFs with MR at higher temperatures resulted in increased levels of solubilization by MR, however, increases in soluble carbohydrate content by subsequent enzymatic hydrolysis decreased as MR temperature increased. Characterization of the MR-solubilized and MR+enzyme-solubilized carbohydrates from quinoa IDF indicated small and linear oligosaccharide structures arising from cellulose, xyloglucans, galacturonans and arabinans. In the case of pearl millet IDF, the MR-solubilized and MR+enzyme-solubilized carbohydrates were mainly constituted by branched arabinoxylans. The optimization of hydrothermal treatments in combination with enzymatic hydrolysis proves to have potential in the generation of fermentable carbohydrates by the solubilization of IDF.

3.2 Introduction

The direct impact of dietary fiber on colonic health is through its fermentative properties (Zhang & Hamaker, 2010b). In quantitative terms, cereals are very important sources of

dietary fiber. However, it must be considered that the majority of fiber from grains is insoluble and generally are not considered to be fermentative. Therefore, some research efforts have focused on finding ways to solubilize the insoluble fiber fraction to make it more fermentable as well as functional. A dietary fiber mixture containing oligo- and polysaccharides with varying degrees of fermentation rate would be particularly desirable, as it would give a constant and extended fermentation throughout the colon. The properties of insoluble dietary fibers (IDF) can be modified through certain types of processing treatments that include physical, hydrothermal, and practical enzymatic applications. Treating IDFs from cereals in order to increase their solubility has been shown to improve its fermentation properties (Bjorck et al., 1984; Drzikova et al., 2005). Some research efforts have focused on finding ways to solubilize IDF to make it more functional and fermentable (Chau et al., 2007; Guillon et al., 1992; Mateos-Aparicio et al., 2010; Napolitano et al., 2009; Nordlund et al., 2013). Based on these ideas, a partial solubilization of enzymatically-isolated IDFs was undertaken in cereal and the pseudocereal grains chosen for this study. IDFs were subjected to hydrothermal treatments in an attempt to generate soluble carbohydrates through break-down of the insoluble polysaccharide matrix for the purpose of enhancing its susceptibility to microbial degradation. The field of biofuels has widely researched the treatment of lignocellulosic biomass for solubilization (Pérez et al., 2008; Weil et al., 1998b; Zhu et al., 2006), and some direction was taken from previous studies in this field. For the more successful treatments, in vitro fecal fermentation patterns of the treated fiber substrates were assessed.

3.3 Materials and Methods

3.3.1 Development of Hydrothermal Treatments of Insoluble Dietary Fibers

Four IDF grain sources were chosen for the development of solubilization treatments, including two pseudocereal grains, quinoa and amaranth, as well as the cereals pearl millet and wheat. IDF samples were subjected to three different hydrothermal treatments (Figure 3.1) for the purpose of solubilizing the fiber and to enhance overall fermentability, even if not solubilized. The hydrothermal treatment methods used were, liquid hot water (LHW), autoclaving (AUT), and microwave radiation treatment (MR) were chosen. IDF suspensions in water (10%, w/v) were prepared, mixed thoroughly and centrifuged. The volume of supernatants was recorded and then supernatants were discarded. For each suspension, water was replenished in the exact amount of supernatant that was removed after centrifugation (to maintain the 10% suspension) and mixed again. These second suspensions were allowed to soak overnight at room temperature.

3.3.1.1 Liquid hot water treatment

Soaked IDF suspensions were transferred into stainless steel tube reactors and then tightly sealed. Reactors were immersed into fluidized sand baths that had been previously equilibrated to 120 °C and were held there for 30 min. After the treatment time had elapsed, reactors were immediately submerged in cold water. Once cooled, treated sample suspensions were centrifuged (8,000 g, 20 min, 15 °C) and 1 mL aliquots of supernatants were taken for measurement of total carbohydrate content. The remainder of the LHW-treated IDF suspension was freeze-dried and ground with a mortar and pestle to a powder that had a particle size ranging from 250-500 μm.

3.3.1.2 Autoclave treatment

Soaked IDF suspensions were transferred into glass tubes and tubes were loosely capped. Tubes containing IDF suspensions were autoclaved for 30 min at high-pressure setting (max temperature 120 °C). After autoclaving and cooling in a cold-water bath, tubes were centrifuged (8,000 g, 20 min, 15 °C). One milliliter aliquots of supernatants were taken for measurement of total carbohydrate content. The remainder of the AUT-treated IDF suspensions was freeze-dried and ground with a mortar and pestle to a powder that had a particle size ranging from 250-500 μm.

3.3.1.3 Microwave radiation treatment

Soaked IDF suspensions were transferred into microwave vessels that contained a magnetic stirring bar. Tightly sealed vessels containing the IDF suspensions were then subjected to microwave radiation, under constant stirring, in the MARSXpress™ microwave (CEM Corporation, Mathews, NC, USA) at 800 W, 120 °C for 30 min. After the treatment time had elapsed and samples were cooled in a cold-water bath, MR-treated suspensions were centrifuged (8,000 g, 20 min, 15 °C) and 1 mL aliquots of supernatants were taken for measurement of total carbohydrate content. The MR-treated IDF suspensions were freeze-dried and ground with a mortar and pestle to a powder that had a particle size ranging from 250-500 μm.

MR treatment was chosen for solubilization trials at higher temperatures. Only quinoa and pearl millet were used as IDF sources in these subsequent trials. MR treatment was applied to 2.5% (w/v) insoluble fiber suspensions in water for 30 min and 800 W of power at 120 °C, 160 °C or 180 °C. Sample suspensions were prepared as previously

described (see section 3.3.1) and solubilized carbohydrate was measured in the 1 mL aliquots of the liquid portion of the suspensions after treatments. Suspensions were freeze-dried and stored until further analysis.

3.3.2 Development of a Sequential Enzymatic Hydrolysis of Hydrothermally-Treated Insoluble Dietary Fibers

In order to determine the conditions of an effective enzymatic solubilization, IDF samples that had been treated with all three hydrothermal methods (LHW, AUT, and MR) were subjected to trials of sequential enzymatic hydrolysis (Figure 3.2). The enzymes were chosen based on the concept of an enzymatic deconstruction of the cell wall polymer matrix. Plant cell walls are composed of cellulose, hemicelluloses, lignin and protein (Albersheim, 1975). In the first trial, incubation with protease was chosen as the first step in the enzyme hydrolysis sequence since protein is thought to reinforce the cell wall structure. In order to help open the cell wall structure, feruloyl esterase was chosen for the second step of the enzymatic treatment because it cleaves diferulic acid bridges between hemicellulose chains and aids in the release of lignin (Faulds & Williamson, 1995; Yu et al., 2002). However, Gamble et al., (2000) reported that phenolic acids released into the medium act as inhibitory compounds to the activity of cellulases and other glycoside hydrolases. In order to remove potential inhibitory hydrolysis products, the medium of the IDF suspensions was changed after each enzyme treatment during the first trial. After fresh medium was added, enzymes to hydrolyze hemicelluloses and cellulose were added.

3.3.2.1 Enzyme Hydrolysis A

In the first trial, referred to as enzyme hydrolysis A, suspensions of treated IDF samples were prepared in deionized-distilled water [1% (w/v)]. Neutral protease was added (0.1 U/g IDF, Sigma P1236) and suspensions were incubated at 50 °C for 4 h with constant agitation and subsequently boiled for 15 min to deactivate the enzyme, centrifuged, and the supernatant collected in a separate container. Water was replenished in the amount of supernatant removed and feruloyl esterase was added (30 U/g IDF, E-FAEZCT Megazyme). Suspensions were incubated at 50 °C for 4 h with constant agitation and subsequently boiled for 15 min to deactivate the enzyme, centrifuged and the supernatant collected in a separate container. Water was replenished again, in the amount of supernatant removed. For cereal IDF suspensions, an enzyme cocktail consisting of endo-β-1,4-xylanase (70 U/g IDF, X2629 Sigma) and cellulase (70 U/g IDF, C2605 Sigma) was added. In case of the pseudocereal IDF suspensions, endopolygalacturonase (70 U/g IDF, E-PGALS Megazyme) was used instead of endoxylanase. Samples were incubated at 50 °C for 8 h with constant agitation and, after 8 h elapsed, they were boiled for 15 min to inactivate the enzymes. Samples were centrifuged, and aliquots of supernatants were collected and analyzed for soluble carbohydrates. The remaining enzyme-treated insoluble dietary fiber residues were freeze-dried.

3.3.2.2 Enzyme Hydrolysis B

To evaluate whether residual protease from the initial protease treatment had a negative effect on the activity of the other enzymes used, a second trial referred to as enzyme hydrolysis B, was conducted. To avoid the proteolysis of subsequent enzymes used, the

procedure was modified to omit the protease treatment and was done without changing the suspension medium after each enzyme.

3.3.2.3 Enzyme Hydrolysis C

The final enzymatic treatment, enzyme hydrolysis C, consisted of 1% (w/v) microwave-treated IDF suspensions from quinoa and pearl millet incubated with protease (0.1 U/g IDF, Sigma P1236) at 50 °C for 4 hours followed by incubation with feruloyl esterase (50 U/g IDF, E-FAERU Megazyme) at 50 °C for 4 hours. IDF suspensions were centrifuged (8,000 g, 20 min, 15 °C) after enzyme treatment and supernatants were collected separately. IDF samples treated with protease and feruloyl esterase were suspended in water again (1%, w/v) and incubated with the same carbohydrate hydrolyzing enzymes (at 100 U/g IDF) used previously. In the case of quinoa IDF, an endo-1,4-β-galactanase (100 U/g IDF, E-GALN Megazyme) was added to the cocktail. Also, incubation time with carbohydrate hydrolyzing enzymes was increased from 8 to 24 h. After the incubations, IDF suspensions were centrifuged (8,000 g, 20 min, 15 °C) and supernatants filtered through vacuum-aided glass Buchner funnels with a medium porosity fritted disc (10-15 μm) to completely remove insoluble fiber particles. Filtered supernatants were collected separately and freeze-dried.

3.3.3 Determination of Soluble Carbohydrate Content

The amount of soluble carbohydrate generated by treatments and enzymatic hydrolysis was quantified by the phenol-sulfuric acid method (Dubois et al., 1956). Aliquots of supernatants from hydrothermally-treated and enzymatically-hydrolyzed samples were diluted 100 times and total carbohydrate content was measured in the diluted samples.

Values for % solubilization were calculated based on soluble carbohydrate content of hydrothermally and enzymatically-treated IDF suspensions. The amount of total carbohydrate (mg CHO/ml) that was solubilized was measured in the supernatant of the suspension and % soluble carbohydrate content was calculated as follows:

$$\text{mg IDF} \times \%\ \text{estimated CHO} = \text{total CHO in suspension}$$

$$\left[{}^{\text{mg CHO}}/_{\text{ml}}\right] \times \text{total vol water in suspension} = \text{total solubilized CHO}$$

$$\left(\frac{\text{total solublized CHO}}{\text{total CHO in suspension}}\right) \times 100 = \%\ \mathbf{solubilized\ CHO}$$

$$\left(\frac{\text{total CHO in suspension} - \text{total solublized CHO}}{\text{total CHO in suspension}}\right) \times 100 = \%\ \mathbf{insoluble\ CHO}$$

3.3.4 Characterization of Solubilized Fiber Oligosaccharides

IDF from quinoa and pearl millet that was solubilized by microwave and enzyme treatments was analyzed for monosaccharide and glycosyl-linkage composition by the methods described by Pettolino et al. (2012). Briefly, monosaccharide composition was analyzed by subjecting samples to a carboxyl reduction and then hydrolyzing into monosaccharides with 2.5 M TFA. Alditol acetates were prepared by reduction with sodium borodeuteride and acetylated with acetic anhydride. For analysis of glycosyl-linkage composition, pre-reduced polysaccharide samples were methylated with iodomethane in a sodium-hydroxide and dimethyl-sulfoxide slurry and methylated polysaccharides were hydrolyzed with 2 M TFA. Partially methylated monosaccharides are then reduced and acetylated to yield partially methylated alditol acetates.

Monosaccharide and glycosyl-linkage compositions were determined by separation, identification and quantification of alditol acetates in acetone with GC-MS.

High-Performance Anion-Exchange Chromatography (HPAEC) was used to analyze fibers that were solubilized after microwave and enzyme treatments for chain length distributions. Two percent solutions of solubilized samples were filtered (0.45μm) and injected into a CarboPac PA-100 pellicular anion-exchange column (Dionex, Sunnyvale, CA) that was pre-equilibrated in a eluent A (150 mM NaOH) at 1.0 mL/min. Chromatographic separation of the oligosaccharides from the sample was achieved by gradient elution from 100% eluent A to 100% eluent B (600 mM sodium acetate in 150 mM NaOH).

3.4 Results and Discussion

3.4.1 Development of a Solubilization Procedure for Insoluble Dietary Fibers

3.4.1.1 Pseudocereals: Quinoa and Amaranth

IDF from amaranth and quinoa were exposed to hydrothermal treatments in order to increase their solubility and enhance susceptibility to enzymatic hydrolysis and fermentation. Three types of treatment were used: LHW, AUT, and MR. The first two are based on the application of heat to suspensions of lignocellulose materials under high-pressure conditions to maintain the water in liquid state (Pérez et al., 2008). MR treatment consists of heating the sample suspension by means of radiation. Microwaves are uniformly absorbed by the suspension, which cause vibration of the molecules that create heat through friction (Mandal et al., 2007).

The untreated amaranth-IDF was 99.6% insoluble and, as is shown in Figure 3.3, all 3 types of treatment resulted in a small increase in amount of soluble carbohydrate. The amount of solubilized carbohydrate was 1.5 and 2.7% with LHW and AUT treatments. Although only minor effect on solubilization was seen here, it has been previously reported that LHW treatment effectively disrupts the lignocellulosic matrix of biomass and partially hydrolyzes hemicelluloses (Weil et al., 1998a; Weil et al., 1998b). Statistical analysis did not show significant differences ($P < 0.05$) among the three treatments.

Enzymatic hydrolysis A of the physically treated amaranth IDFs had a substantial effect on solubilization (Figure 3.3). Soluble carbohydrate contents ranged from about 38.4 to 53.9% after the combination of treatments. Both LHW and AUT in combination with enzyme hydrolysis A resulted in the high solubilization for amaranth IDF and were significantly higher than the MR treatment and enzyme hydrolysis A combination.

Conversion of insoluble to soluble dietary fiber, from the different treatments, was even more notable in quinoa IDF (initially 99.6% insoluble) (Figure 3.4). For the physical treatment alone the solubilization effect was still low, but higher than for amaranth IDF. MR and LHW treatments resulted in the highest amounts of solubilization (~5.1%). AUT had a lower effect (1.7% increase) and was comparable to amaranth IDF solubilization levels. In preparation for treatment, quinoa-IDF held more water[2] after

[2] Water held by IDF samples is described here in terms of the volume of supernatant after centrifugation of suspension (10% w/v) at 10,000 g for 30 min subtracted from the total volume of water in suspension.

overnight soaking and had a more noticeable increase[3] in viscosity after treatment, than amaranth IDF. Since the principle of MR treatment is based on how the solvent absorbs microwave energy and passes it on as heat to the surrounding molecules (Mandal et al., 2007), it can be hypothesized that if quinoa IDF has better hydration properties, the effect of MR treatment, as well as its susceptibility to other hydrothermal treatments, would be greater. Solubilization by enzymatic hydrolysis A of hydrothermally-treated quinoa IDFs was higher than amaranth IDF and ranged from 32.9 to 46.8% soluble fiber. Treated quinoa IDF responded in a different way to enzymatic hydrolysis compared to amaranth. MR-treated quinoa IDF resulted in a significantly higher amount of soluble carbohydrate after enzymatic hydrolysis A; the opposite was the case for MR-treated amaranth IDF.

3.4.1.2 Cereals: Pearl Millet and Wheat

Pearl millet and wheat IDFs were also subjected to hydrothermal treatments followed by enzymatic hydrolysis A. Soluble carbohydrate content of treated cereal-IDFs is presented in Figures 3.5 and 3.6. For the hydrothermal treatments alone, solubilization of pearl millet IDF ranged from only 1.1 to 1.5% with somewhat higher amounts obtained from LHW and MR. Pearl millet IDF has around 17% lignin content (Table 2.3) and that may be a major hindrance for solubilization by hydrothermal treatments under the mild conditions used here. Studies on the treatment of highly lignified biomass such as wood chips or straw report that higher temperatures, pressure, treatment time or power[4], are needed than the ones used here (Guillon et al., 1992; Hu & Wen, 2008; Wang et al., 2008; Zhu et al., 2006). Thus, there might still be a potential for effecting increased

[3] From observation only, quantitative determinations of viscosity were not made.
[4] Watts for microwave radiation.

solubilization or degradation of the insoluble polysaccharide matrix for improved fermentability through use of more rigorous hydrothermal treatment conditions.

Enzymatic hydrolysis A increased the amount of soluble carbohydrate from pearl millet IDF, but the increase was much lower than that for the pseudocereal IDFs. Solubilization of pearl millet IDF after enzymatic hydrolysis A was ~14.0% and the combination with MR treatment resulted in the highest amount. In comparison with wheat IDF, which had significantly higher soluble carbohydrate content after enzymatic hydrolysis (~21.0%), the higher lignin content in pearl millet IDF may also here be a greater hindrance for enzymatic hydrolysis, because lignin forms a protective barrier around carbohydrate polymers that hinders enzyme attack (Öhgren et al., 2007). Thus, optimization of treatments for disruption of lignocellulosic matrix in IDFs is very important. Further enzymatic hydrolysis of hydrothermally-treated cereal IDFs generated increased amounts of soluble carbohydrate, though were much lower than for the same treated pseudocereal IDFs. Such treated IDF for wheat gave higher soluble product than for pearl millet.

3.4.1.3 Enzymatic Hydrolysis without Protease Treatment

Insoluble dietary fibers that were previously treated with MR treatment were subjected to enzymatic hydrolysis B, which did not include the initial protease step. MR treatment was chosen because it had resulted in significantly higher solubilization with the combination of treatments for the majority of IDFs used. Figure 3.7 shows the soluble carbohydrate content for quinoa and amaranth IDF samples. Untreated IDFs from both pseudocereals were 99.6% insoluble; after microwave treatment, quinoa and amaranth IDF had 4.8 and 1.5% soluble carbohydrate content, respectively. This increased to 13.0%

for both IDFs after enzyme hydrolysis B, which was significantly less than the increase generated by enzyme hydrolysis A (38.4% amaranth IDF, 46.8% quinoa IDF). In case of the cereal IDFs (untreated, 100% insoluble), MR treatment resulted in only 1.1 and 1.8% solubilization of carbohydrates for pearl millet-IDF and wheat-IDF, respectively. As is shown in Figure 3.8, further solubilization by enzyme hydrolysis B produced an increase to 11.8% for pearl millet IDF and 18.0% for wheat IDF. Similar to results from enzyme hydrolysis A, wheat IDF was more susceptible than pearl millet to solubilization by enzymes.

Notably, enzyme hydrolysis B produced considerably less soluble carbohydrates in all samples than enzyme hydrolysis A. Thus, protease treatment was an important step in increasing soluble fiber content. Also, it did not appear to have a negative effect on the activity of the esterase and glycoside hydrolases. Besides the use of a protease as the first step of enzymatic solubilization, enzyme hydrolysis A also included a change to fresh dispersion medium[5] after each enzyme, and may also have been a determining factor in the resulting differences in soluble fiber content. Change of medium was done in order to avoid the inhibitory effects of degradation products from previous enzymatic hydrolysis on the activity of the next enzyme. Thus, the lower solubilization that resulted from enzyme hydrolysis B could have been due to two factors: 1) lower susceptibility of IDFs to glycoside hydrolases because of the presence of protein, or 2) decreased activity of glycoside hydrolases caused by the inhibitory effects of degradation products from feruloyl esterase (i.e., ferulic acid or lignan).

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[5] IDF samples were dispersed in deionized-distilled water for treatment and enzyme hydrolysis.

3.4.1.4 Microwave Radiation Treatment at Higher Temperatures

MR treatment was chosen for solubilization trials at higher temperatures. Only quinoa and pearl millet were used in these trials. MR treatment was applied to 10% (w/v) insoluble fiber suspensions for 30 min at 120 °C, 160 °C and 180 °C and results are presented in Figures 3.9 and 3.10. For both quinoa and pearl millet, solubilization of insoluble fiber increased with increasing temperature of MR treatment. Increases in solubilization were greater for quinoa whose soluble carbohydrate content increased from 4.8% at 120 °C to 11.0% at 160 °C and 26.0% at 180 °C. On the other hand, increases in soluble carbohydrate content were low for pearl millet after MR treatment at 120 °C and 160 °C (1.0% and 1.6%, respectively), but increased to 8.0% with treatment at 180 °C. It is evident that pearl millet requires higher temperature conditions to effect significant solubilization. LHW treatments, which are commonly used in wood chips and other highly lignified materials and can reach temperatures of 200 °C or higher, perhaps could result in greater solubilization of insoluble fibers from pearl millet IDF and other cereals.

When MR treatments at each temperature were combined with enzyme hydrolysis A, an interesting trend was observed. As shown in Figures 3.9 and 3.10, increasing temperatures of MR treatment had an inverse effect on solubilization by enzyme hydrolysis. Studies that report on the optimization of hydrothermal treatments of biomass indicate that higher temperatures promote lignin and sugar degradation which results in the formation of compounds that have detrimental effects on activities of the enzymes used subsequently (Alvira et al., 2010; Oliva et al., 2003; Sun & Cheng, 2002). This might explain why this trend was more discernible for pearl millet, since it contains

a significantly higher amount of lignin, 17.0% versus 9.0% in quinoa IDF. These results further indicate that maximum solubilization of IDF from hydrothermal treatments and enzymatic hydrolysis warrants a proper optimization that involves all factors of treatments such as temperature, treatment time, pressure (LHW) or wattage (MR), and total solids content of the suspensions.

3.4.2 Final Solubilization Treatments for Insoluble Dietary Fibers

After trials for the development of an effective solubilization procedure for insoluble dietary fibers from cereals and pseudocereals, the final procedure consisted of MR treatment at 180 °C for 30 min for 2.5% (w/v) IDF suspensions followed by an extensive enzymatic hydrolysis process. As described in section 3.3.2, the final enzymatic hydrolysis included an additional enzyme for quinoa IDF and increased incubation time for the carbohydrate hydrolyzing enzymes. The amounts of soluble carbohydrates generated from this final procedure (Figures 3.11 and 3.12) were higher than those obtained with microwave and enzyme treatments during the development trials. Quinoa IDF suspensions had 34.0% soluble carbohydrate content after MR treatment and it increased to 48.0% after the MR-treated IDF was enzymatically hydrolyzed. In the case of pearl millet IDF suspensions, MR treatment at 180 °C generated 8.0% soluble carbohydrate content that increased to 11.8% with subsequent enzymatic hydrolysis. Higher levels of solubilization were not reached for pearl millet under the conditions used in the final solubilization treatments.

3.4.3 Characterization of Carbohydrates Solubilized by Final Solubilization Treatments

As reported above, IDFs isolated from quinoa and pearl millet whole grain flours were subjected to MR treatment and enzymatic hydrolysis to effect solubilization in order to improve their fermentable properties. Treatments resulted in a range of enhanced soluble fiber substrates that contained a variety of oligosaccharides differing in composition and structure. Solubilized fractions were first analyzed for monosaccharide and glycosyl-linkage composition (Table 3.1 and 3.2). MR-solubilized fiber from quinoa IDF was mainly composed of glucose, galacturonic acid, arabinose and xylose. In general, glycosyl-linkage data suggests that MR treatment only of quinoa IDF solubilized portions of cellulose, homogalacturonans and RG-I, arabinan, and xyloglucan polymers. The combination of MR+enzyme treatments mainly solubilized cellulose, and xyloglucans with galactose side chains. Although an endo-polygalacturonase was used in the sequential enzymatic hydrolysis of quinoa IDF, galacturonic acid was not found in the monosaccharide composition of the soluble fiber generated from its IDF.

Monosaccharide and glycosyl-linkage composition of MR-solubilized and MR+enzyme-solubilized fibers from pearl millet IDF is presented in Table 3.14. As opposed to quinoa IDF where treatments mainly solubilized cellulose; treatment of pearl millet IDF mainly solubilized arabinoxylan. MR treatment generated soluble arabinoxylan that were highly branched with arabinose and galactose. MR+enzyme treatment solubilized arabinoxylans with a slightly lower branching degree. In addition to arabinoxylans, treatments of pearl millet IDF also solubilized a small portion of cellulose.

HPAEC analysis indicated that the soluble fractions from quinoa obtained after MR and MR+enzyme treatments mainly contained mono-, di-, and trisaccharides, but smaller proportions of larger oligosaccharides were also observed (Appendix 3A). In the case of MR-solubilized and MR+enzyme solubilized fractions from pearl millet, mono-, di-, and trisaccharides were the main constituents. Solubilization of insoluble dietary fibers from quinoa and pearl millet by means of MR treatment and enzyme hydrolysis resulted in a variety of oligosaccharides. These soluble oligosaccharides in the treated substrates now constitute fiber substrates of improved fermentability than their untreated counterparts. In addition, the range of polysaccharide structures within each fiber substrate may result in extended fermentation patterns with improved short chain fatty acid profiles.

3.5 Conclusions

Although solubilization of IDFs by hydrothermal treatments alone was generally low, ranging from 0.4 to 5.1% where LHW and MR resulted in the higher levels, the combination of those treatments with enzyme hydrolysis generated substantial amounts of soluble fibers from IDFs. Enzyme hydrolysis of hydrothermally-treated fibers that included protease caused the greatest increase in solubilization. Pseudocereals were more susceptible to solubilization by the combination of hydrothermal and enzymatic treatments resulting in ~32.9-53.9% solubilization of fiber. Cereal samples did not respond as effectively, with only ~13.6-22.3% maximum solubilization achieved. In addition to solubilizing considerable portions of the IDFs, the dietary fiber fractions that remained insoluble after treatments might have increased susceptibility to microbial degradation as the cell wall polymer matrix that constitutes insoluble fiber likely becomes

more porous and or less recalcitrant due to weakening of the lignin and crystalline cellulose structures in the cell wall matrix. MR treatment applied at higher temperatures, however, had an inverse effect on solubilization levels achieved by subsequent enzyme hydrolysis. Lignin and sugar degradation products generated at high temperatures could have possibly inhibited or reduced the activity of the carbohydrate hydrolyzing enzymes used.

The substantially solubilized fiber substrates made from quinoa IDF after treatments contained amounts of soluble carbohydrate that ranged from 32.9 to 46.8%. Fiber substrates from pearl millet had lower soluble carbohydrate contents ranging from 8.3 to 23.4%. Treatments of insoluble dietary fibers resulted in a range of treated fiber substrates (mixed insoluble/soluble fiber preparations) that contained a variety of oligosaccharides differing in composition and structure. Highly branched oligosaccharides, mainly arising from arabinoxylans, were solubilized from pearl millet IDF. In the case of quinoa IDF, oligosaccharides from cellulose and pectic polysaccharides were solubilized in comparable amounts. These results show that IDFs, which are usually found in by-products of cereal (or pseudocereal) processing, can be sources of fiber substrates with high fermentability and a greater degree of complexity with potential health benefits different than those of prebiotics currently available, which are constituted by a single type of carbohydrate structure (i.e., FOS, GOS, AXOS), tend to be rapidly fermented, and often generate discomfort due to high gas production.

Table 3.1 Monosaccharide and glycosyl-linkage composition of MR-solubilized and MR+enzyme[a] solubilized fiber from quinoa[b] insoluble dietary fiber

Monosaccha-ride	Microwave SDF	Microwave & Enzyme SDF	Glycosyl-Linkage	Microwave SDF	Microwave & Enzyme SDF
	mol %			mol %	
Glucose	31.5 ± 0.02	44.5 ± 0	1→4-Glc*p*	31.5 ± 0.02	44.5
Galacturonic Acid	26.7 ± 0.02		1→4-GalA	26.7 ± 0.015	
Arabinose	13.2 ± 0.01		1→5-Ara*f*	13.2 ± 0.01	
Galactose	9.9 ± 0	36.9 ± 0	1→6-Gal*p*	9.9	
Xylose	9.7 ± 0	18.5 ± 0.01			
Rhamnose	6.7 ± 0.01		1→2,4-Rha*p*	6.7 ± 0.005	
Fucose	5.9 ± 0		Terminal →	5.9	

[a]Enzyme hydrolysis C, which includes protease → feruloyl esterase → cellulase, endopolygalacturonase, β-galactanase, was used in combination with microwave radiation at 180 °C as the final procedure to solubilize quinoa insoluble dietary fiber.
[b]Only quinoa insoluble dietary fiber was used for the final trials to make the # of samples used more manageable. Amaranth insoluble dietary fiber was excluded.

Table 3.2 Monosaccharide and glycosyl-linkage composition of MR-solubilized and MR+enzyme solubilized fiber from pearl millet insoluble dietary fiber

Monosaccha-ride	Microwave SDF	Microwave & Enzyme SDF	Glycosyl-Linkage	Microwave SDF	Microwave & Enzyme SDF
	mol %			mol %	
Arabinose	50 ± 0	26 ± 0	Terminal-Ara*f*→	32 ± 0.002	
			1→2-Ara*f*	3 ± 0.001	
			1→3-Ara*f*	7 ± 0.002	
			1→5-Ara*f*	9 ± 0.009	
Xylose	30.2 ± 0.01	37 ± 0	Terminal-Xyl*p*→	5 ± 0.002	
			1→4-Xyl*p*	10 ± 0.004	8 ± 0.004
			1→3,4-Xyl*p*	12 ± 0.004	13 ± 0.009
			1→2,3,4-Xyl*p*	3 ± 0.001	17 ± 0.01
Glucose	15 ± 0	26.5 ± 0.02	Terminal-Glc*p*→	5 ± 0.001	11 ± 0.008
			1→4-Glc*p*	10 ± 0.001	15 ± 0.005
Galactose	4.8 ± 0	10.5 ± 0.05	Terminal-Gal*p*→	5 ± 0.001	
A/X	1.65	0.7			

[a]Enzyme hydrolysis C, which includes protease → feruloyl esterase → cellulase and endoxylanase, was used in combination with microwave radiation at 180 °C as the final procedure to solubilize pearl millet insoluble dietary fiber.
[b]Only pearl millet insoluble dietary fiber was used for the final trials to make the # of samples used more manageable. Sorghum insoluble dietary fiber was excluded.

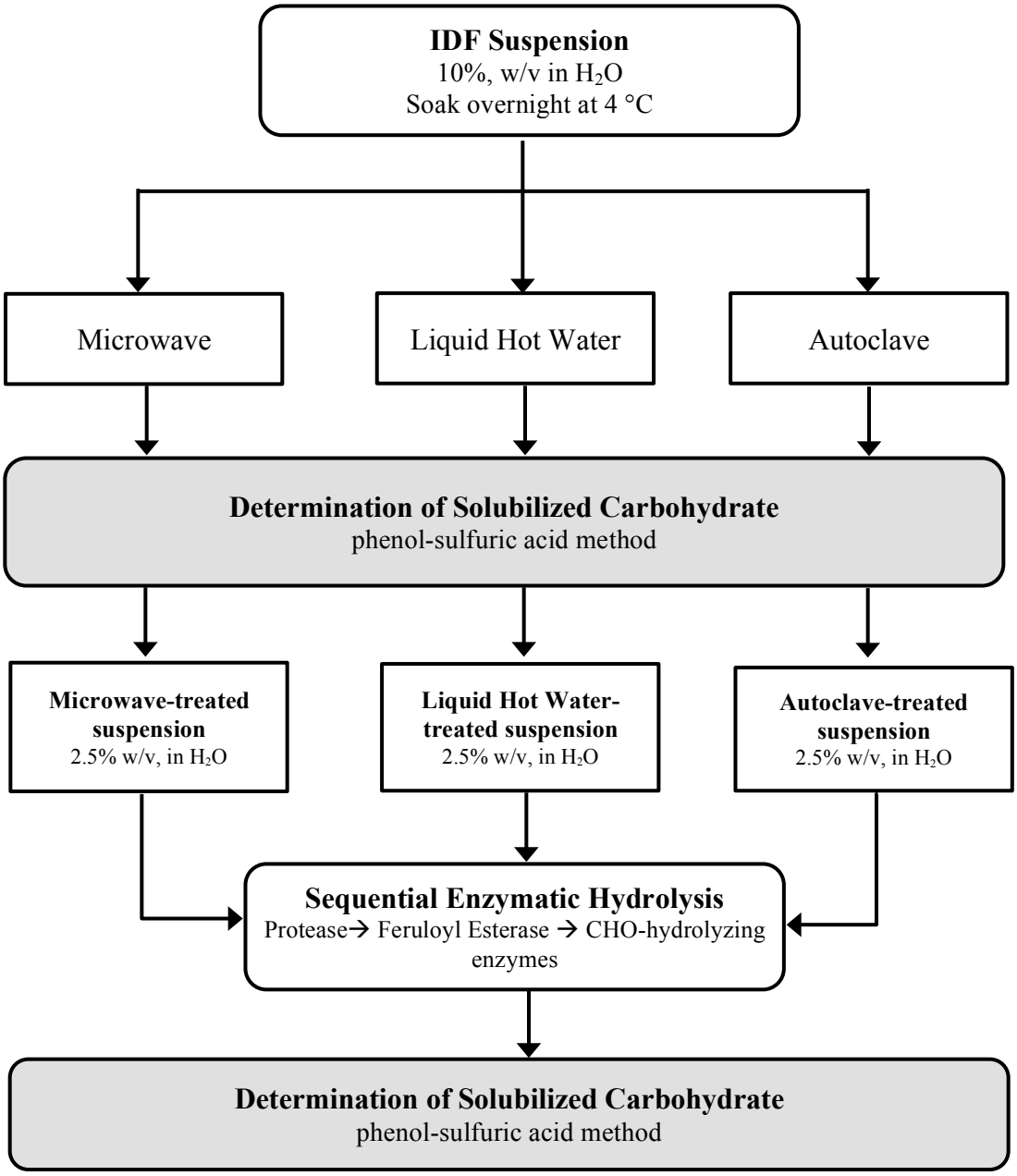


Figure 3.1 Procedure for microwave, liquid hot water, and autoclave treatments followed by enzymatic hydrolysis for solubilization of insoluble dietary fibers from cereal and pseudocereal grains.

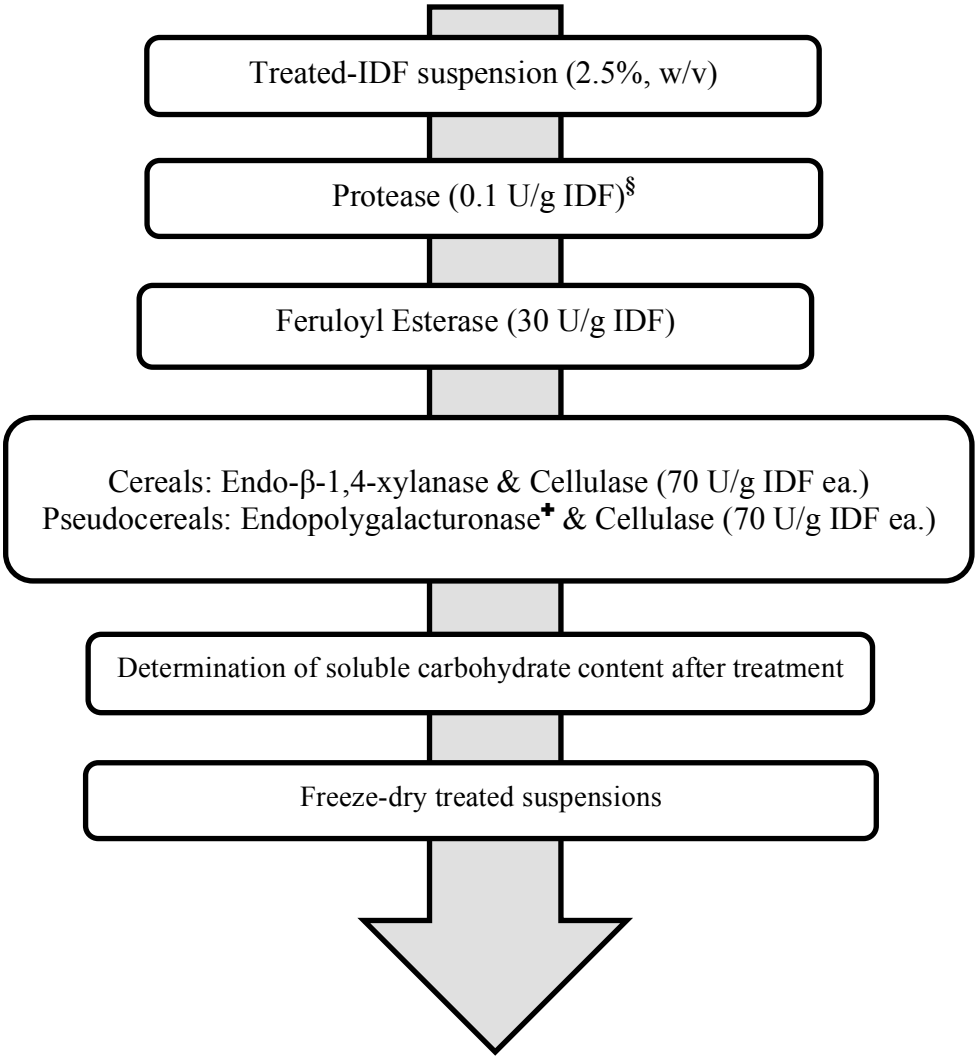


Figure 3.2 Diagram of sequential enzymatic hydrolysis A for the solubilization of LHW, AUT, and MR-treated insoluble dietary fibers from quinoa and pearl millet.

§Protease step is not included in enzyme hydrolysis B.
✚For enzyme hydrolysis C, an endo-1,4-β-galactanase (at 100 U/g IDF) was added to the enzyme cocktail used to hydrolyze quinoa IDF.

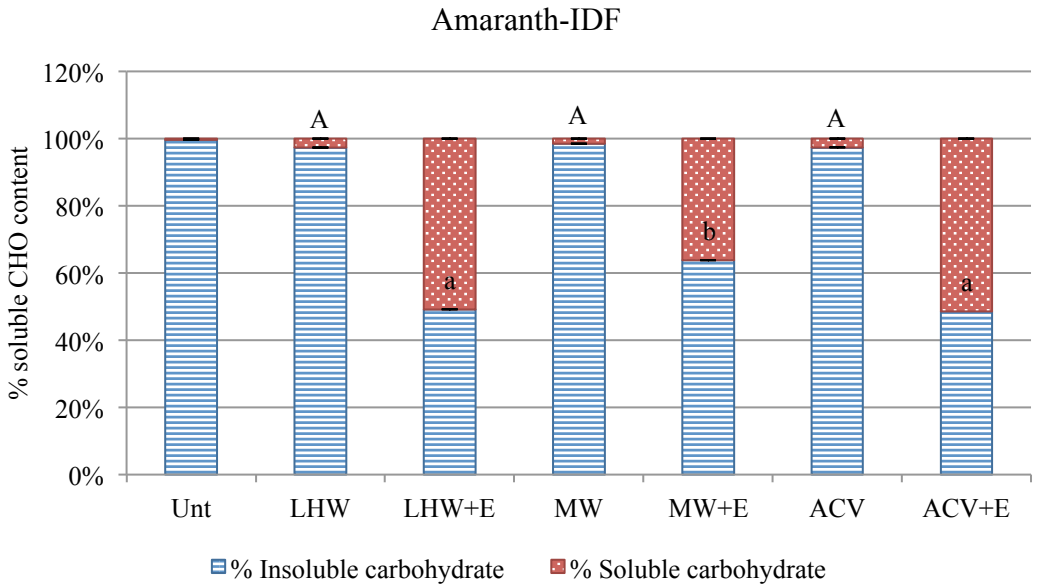


Figure 3.3 Insoluble and soluble carbohydrate content of water-suspended amaranth IDF after hydrothermal treatments and enzymatic hydrolysis A. Abbreviations: Unt = untreated IDF; LHW = liquid hot water treated IDF; MW = microwave treated IDF; ACV = autoclave treated IDF; E = enzymatically hydrolyzed IDF. Error bars show standard deviation (bars may not be appreciable enough to see). Significant differences ($P < 0.05$) are shown as letters above each column; upper case letters indicate differences between pretreatments; lower case letter indicate differences between treatments + enzyme hydrolysis A. Values are the averages of duplicate measurements.

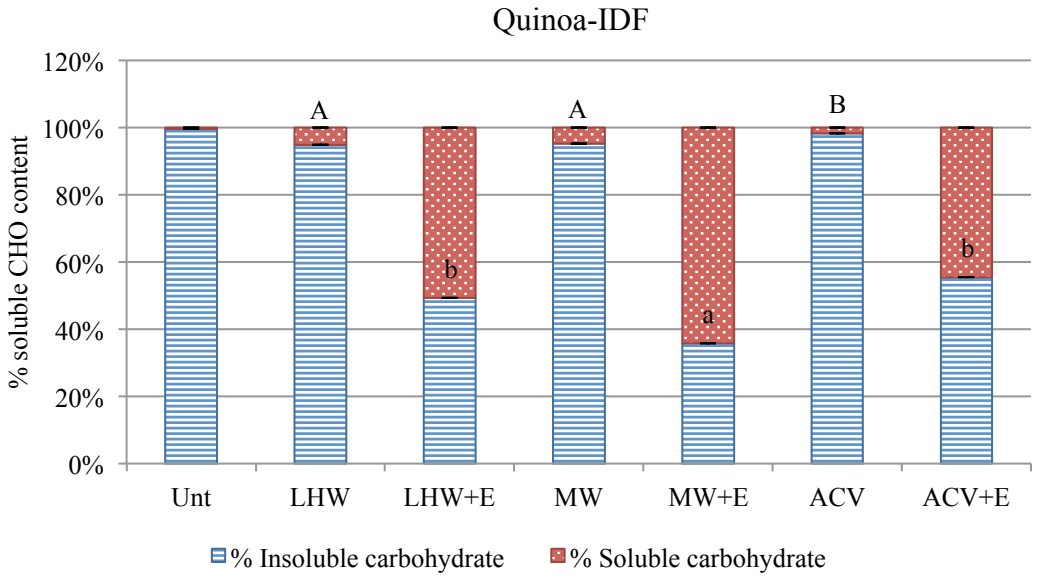


Figure 3.4 Insoluble and soluble carbohydrate content of water-suspended quinoa IDF after hydrothermal treatments and enzymatic hydrolysis A. Abbreviations: Unt = untreated IDF; LHW = liquid hot water treated IDF; MW = microwave treated IDF; ACV = autoclave treated IDF; E = enzymatically hydrolyzed IDF. Error bars show standard deviation (bars may not be appreciable enough to see). Significant differences ($P < 0.05$) are shown as letters above each column; upper case letters indicate differences between pretreatments; lower case letter indicate differences between pretreatments + enzyme treatment A. Values are the averages of duplicate measurements.

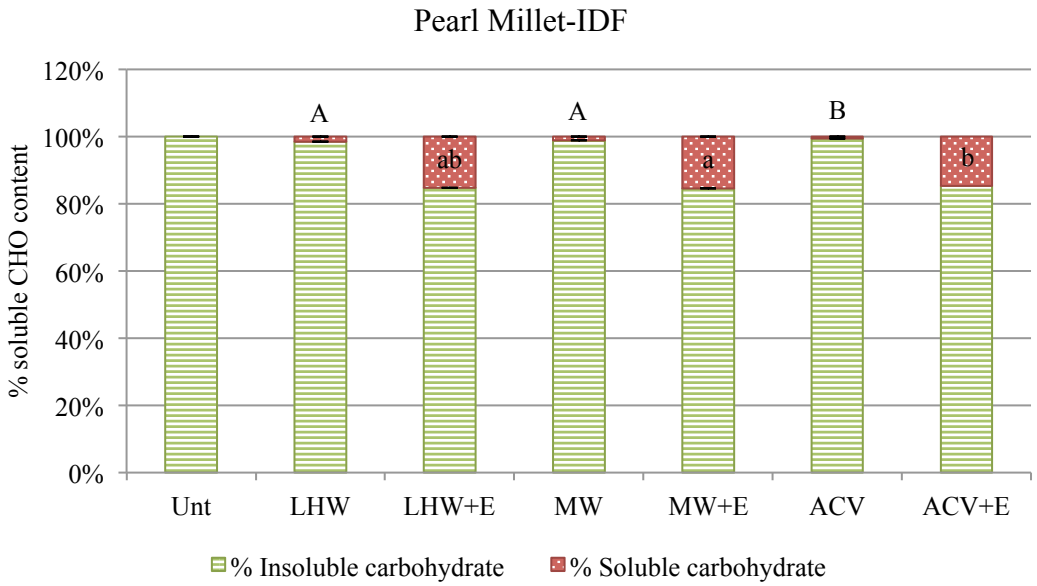


Figure 3.5 Insoluble and soluble carbohydrate content of water-suspended pearl millet IDF after hydrothermal treatments and enzymatic hydrolysis A. Abbreviations: Unt = untreated IDF; LHW = liquid hot water-treated IDF; MW = microwave-treated IDF; ACV = autoclave-treated IDF; E = enzymatically hydrolyzed IDF. Error bars show standard deviation (bars may not be appreciable enough to see). Significant differences ($P < 0.05$) are shown as letters above each column; upper case letters indicate differences between pretreatments; lower case letter indicate differences between pretreatments + enzyme treatment A. Values are the averages of duplicate measurements.

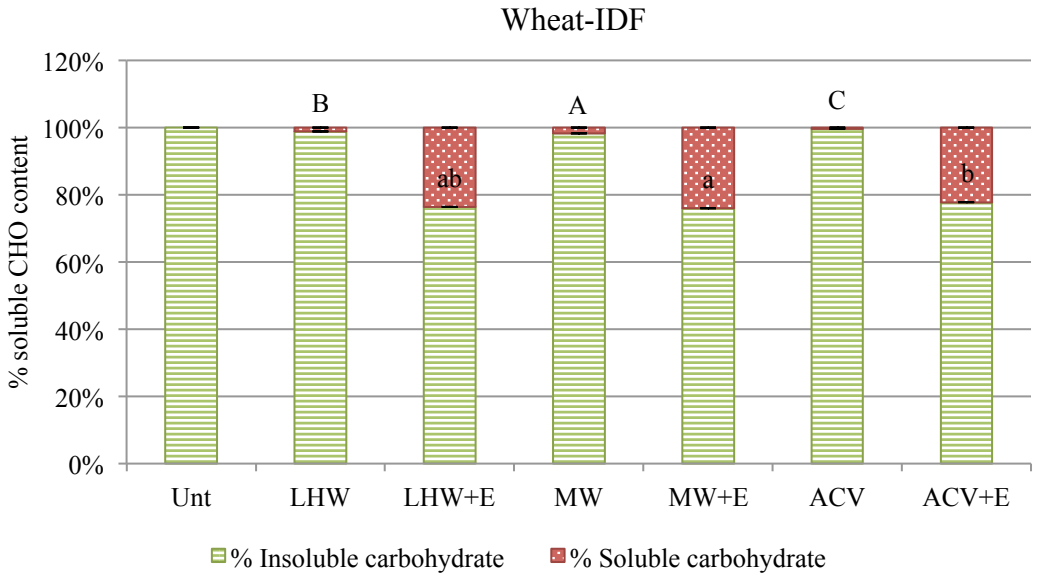


Figure 3.6 Insoluble and soluble carbohydrate content of water-suspended wheat IDF after hydrothermal treatments and enzymatic hydrolysis A. Abbreviations: Unt = untreated IDF; LHW = liquid hot water-treated IDF; MW = microwave-treated IDF; ACV = autoclave-treated IDF; E = enzymatically hydrolyzed IDF. Error bars show standard deviation (bars may not be appreciable enough to see). Significant differences ($P < 0.05$) are shown as letters above each column; upper case letters indicate differences between pretreatments; lower case letter indicate differences between pretreatments + enzyme treatment A. Values are the averages of duplicate measurements.

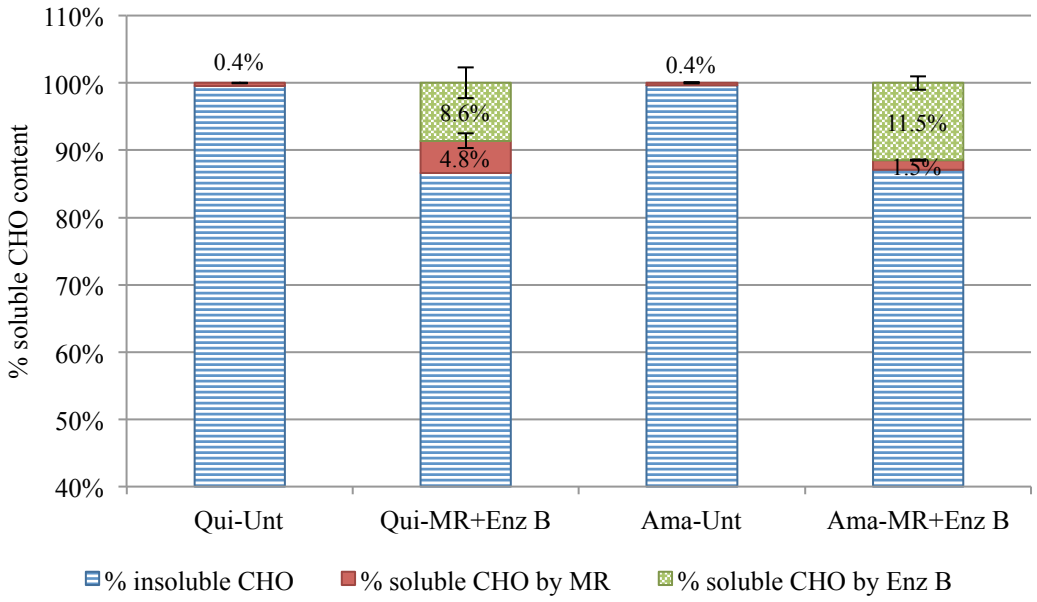


Figure 3.7 Insoluble and soluble carbohydrate content of pseudocereal IDFs after microwave treatment and enzyme hydrolysis B. Abbreviations: Qui= quinoa-IDF; Unt = untreated IDF; MW = microwave-treated IDF; MW+E = microwave and enzyme-treated IDF; Ama = amaranth IDF; error bars show standard deviation (some bars may not be appreciable enough to see); % total soluble carbohydrate content is shown above the columns for microwave + enzyme treatment B. Values are the averages of duplicate measurements.

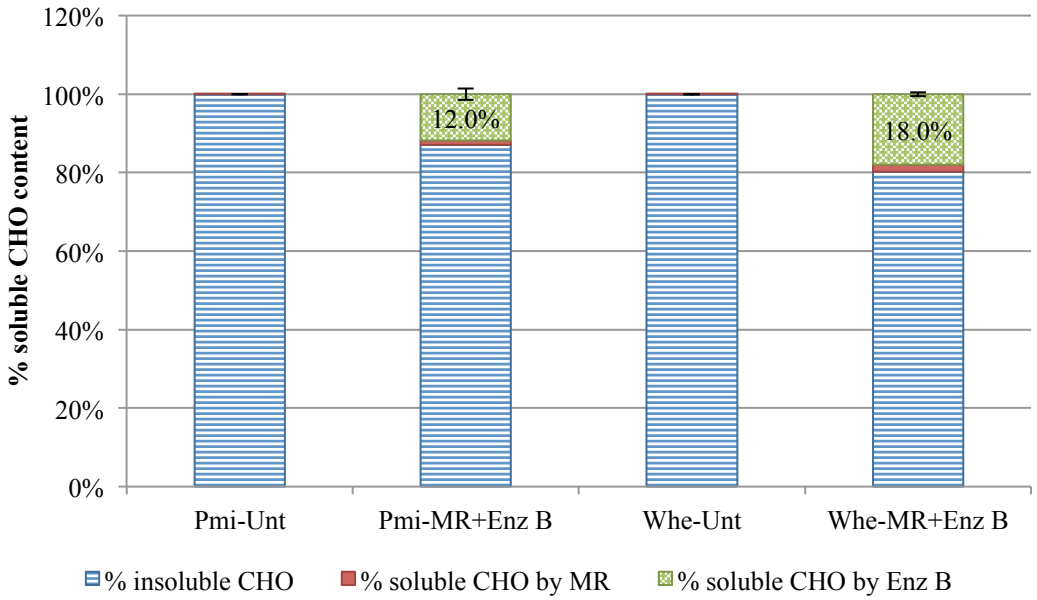


Figure 3.8 Insoluble and soluble carbohydrate content of cereal IDFs after microwave treatment and enzyme hydrolysis B. Abbreviations: Pmi = pearl millet-IDF; Unt = untreated IDF; MW = microwave-pretreated IDF; MW+E = microwave and enzyme treated IDF; Whe = wheat-IDF; error bars show standard deviation (bars may not be appreciable enough to see); % total soluble carbohydrate content is shown above the columns for microwave + enzyme treatment B. Values are the averages of duplicate measurements.

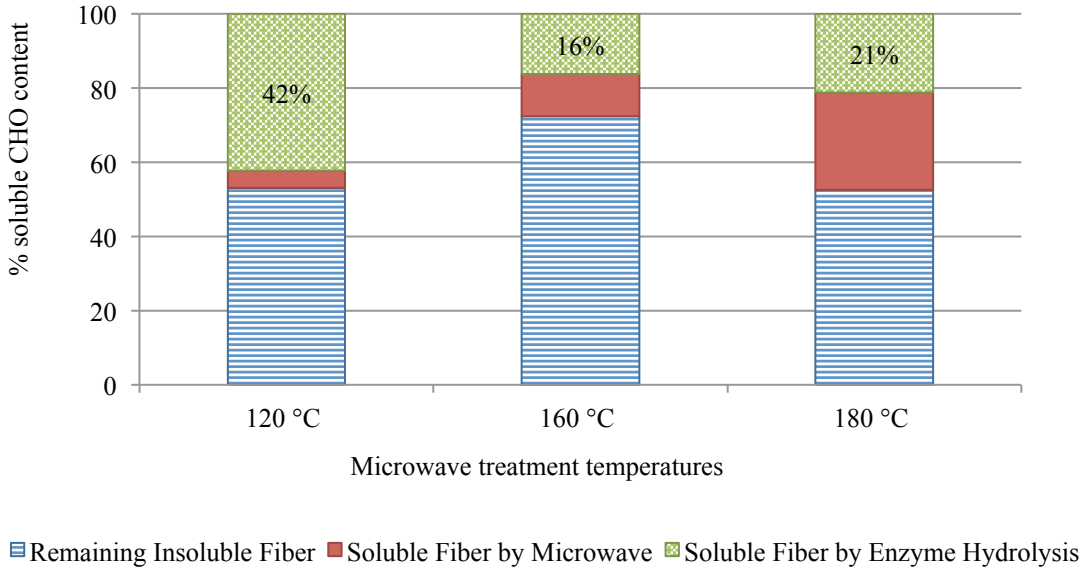


Figure 3.9 Insoluble and soluble carbohydrate content of quinoa IDFs after microwave treatment at 120 °C, 160 °C, and 180 °C in combination with enzyme hydrolysis A. Percent total soluble carbohydrate content by enzyme hydrolysis is shown in the corresponding columns. Increases in microwave radiation treatment temperature resulted in higher amounts of microwave-solubilized fiber but decreased enzyme-solubilized fiber. Values are the averages of duplicate measurements.

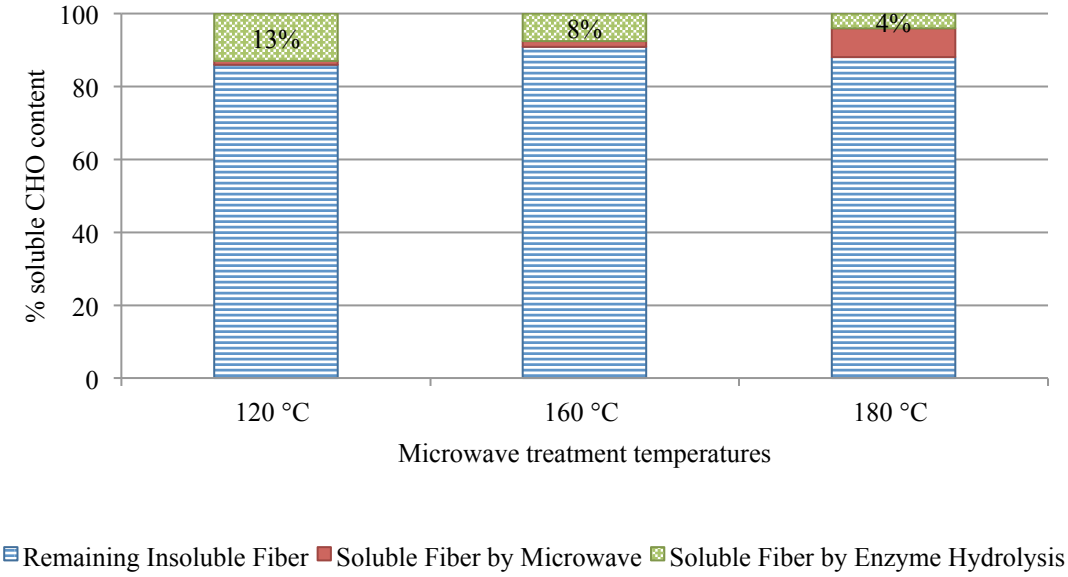


Figure 3.10 Insoluble and soluble carbohydrate content of pearl millet IDFs after microwave treatments at 120 °C, 160 °C, and 180 °C in combination with enzymatic hydrolysis A. Percent total soluble carbohydrate content by enzyme hydrolysis is shown in the corresponding columns. Increases in microwave radiation treatment temperature resulted in higher amounts of microwave-solubilized fiber but decreased enzyme-solubilized fiber. Values are the averages of duplicate measurements.

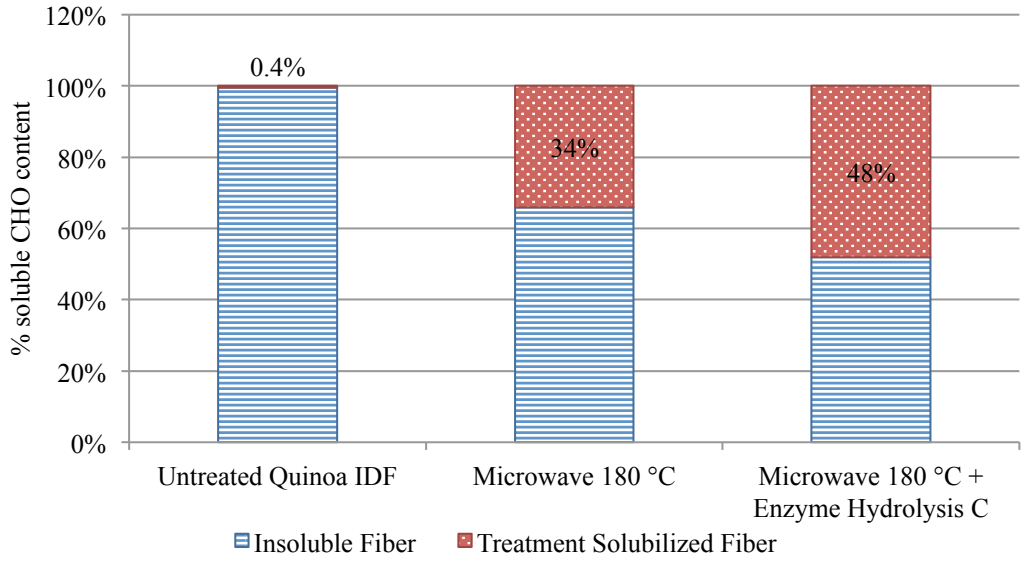


Figure 3.11 Insoluble and soluble carbohydrate content of water-suspended quinoa IDF (2.5%, w/v) after microwave treatment at 180 °C for 30 min and enzyme hydrolysis C. Percent total soluble carbohydrate content by each treatment or treatment combination is shown in the corresponding columns. Microwave treatment alone solubilized 34% of the quinoa IDF; when combined with enzymatic hydrolysis C, 48% of the quinoa IDF was solubilized. Values are the averages of duplicate measurements.

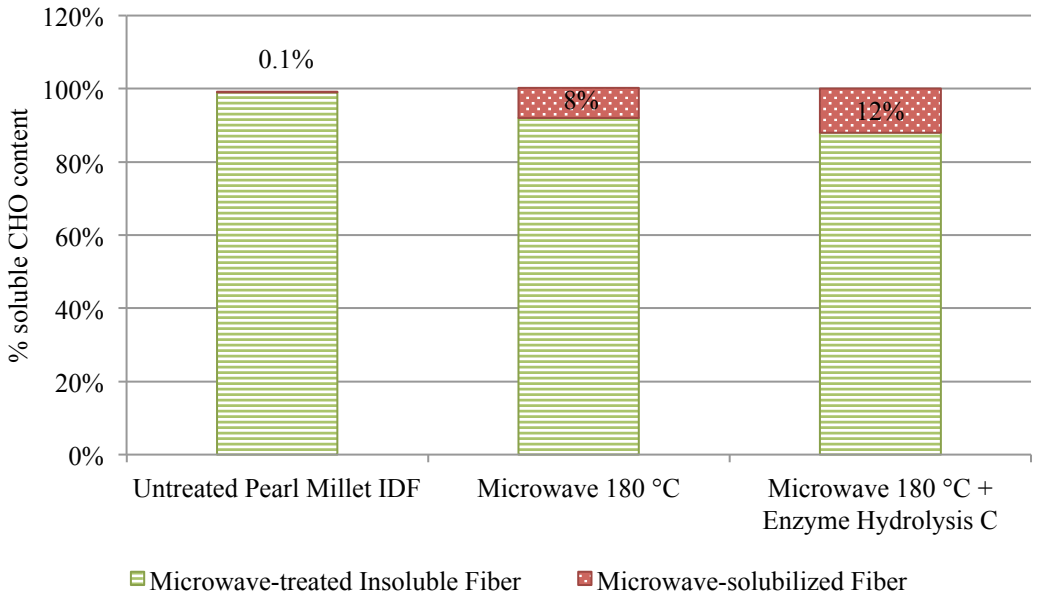


Figure 3.12 Insoluble and soluble carbohydrate content of water-suspended pearl millet IDF (2.5%, w/v) after microwave treatment at 180 °C for 30 min and enzyme hydrolysis C. Percent total soluble carbohydrate content by each treatment or treatment combination is shown in the corresponding columns. Microwave treatment alone solubilized 8% of the pearl millet IDF; when combined with enzymatic hydrolysis C, 12% of the pearl millet IDF was solubilized. Values are the averages of duplicate measurements.

CHAPTER 4. MODIFICATION OF INSOLUBLE DIETARY FIBERS BY MICROWAVE AND ENZYMATIC TREATMENTS IMPROVES *IN VITRO* FECAL FERMENTATION PROPERTIES

4.1 Abstract

Insoluble dietary fibers have poor fermentability and their main contribution to health is attributed to increased stool bulking and reduction in colonic transit time. The purpose of this research was to improve the fermentability of insoluble dietary fibers isolated from quinoa and pearl millet whole grain flours. Insoluble dietary fibers were subjected to treatments with microwave radiation and hydrolyzed with a series of enzymes in order to increase fiber solubility and susceptibility to microbial degradation. These treatments generated a variety of fiber substrates with varying soluble fiber contents and notably improving the fermentability of fiber materials that remained insoluble after treatments. The resulting fibers, then, were composed of three types of fiber insoluble-non-fermentable, insoluble-fermentable, and soluble-fermentable. The soluble-fermentable fiber fraction was predominant in treated fiber substrates from quinoa. Treated substrates from pearl millet mainly contained insoluble-fermentable fiber. In general, treated fiber substrates had significantly higher gas and short chain fatty acid (SCFA) production than the untreated total dietary fibers. Although microwave treatment alone had a significant effect in increasing fermentability, its combination with enzymatic hydrolysis resulted in

more substantial improvements in fermentability in terms of gas and total SCFA production, as well as in higher butyrate concentrations. Fiber substrates from quinoa fermented at a faster rate than substrates from pearl millet, most likely due to the higher amount of soluble-fermentable fiber it contained. SCFA production overall was comparable between the two types of fiber.

4.2 Introduction

Several of the beneficial effects of dietary fibers have been related to their microbial fermentation in the colon (Karpinnen et al., 2000). Short chain fatty acids, which are the main products of fiber fermentation in the colon, inhibit the growth of pathogenic bacteria, reduce the production of secondary bile acids, increase mineral absorption, and provide energy to colonic epithelial cells (Galvez et al., 2005; Scholz-Ahrens et al., 2007; Thornton, 1981). Thus, a lot of attention has been paid to fermentable dietary fibers, particularly the soluble fibers. Accordingly, the main goal of this project was to find methods to increase soluble fiber content through conversion of insoluble to soluble fibers. Also, although it is generally assumed that insoluble dietary fibers are poorly fermented and that their main contribution is to fecal bulking and reduced transit time (Bach Knudsen et al., 1997), insolubility does not always translate into lack of fermentability (e.g., resistant starch). Thus, there was a focus on the effect of physical processing on increasing fermentability of the still insoluble fiber fraction after treatment.

Another perhaps less obvious, but still potentially important, aspect of soluble fiber fermentation relates to rate and location of fermentation in the colon. Because many soluble fibers (e.g., inulin) are readily degraded by intestinal microbiota, their fast

fermentation rate can also cause tolerability issues and causes a deficit of carbohydrate substrate in more distal regions of the colon (Cummings et al., 2001). Considering the lack of fermentable substrate and increased production of toxins from putrefactive fermentation in the distal colon that is more prone to disease (Lim et al., 2005), it is desirable that a dietary fiber substrate can sustain an extended production of SCFAs mediated through prolonged microbial fermentation into the distal colon (Rose et al., 2010b). A low initial rate of gas production avoids abdominal discomfort caused by increased bloating and flatus frequency (Cummings et al., 2001; Kaur et al., 2011). A dietary fiber substrate that constitutes a variety of carbohydrates with a range of structures and different degrees of solubility may result in constant and extended fermentation throughout the colon. Thus, fiber substrates produced from quinoa and pearl millet insoluble fibers using microwave and enzymatic treatments, which varied in amounts of soluble fiber and insoluble fiber, were evaluated for their fermentative properties.

4.3 Materials and Methods

4.3.1 Preparation of Treated Fiber Substrates

Suspensions of insoluble dietary fibers (IDF) from quinoa and pearl millet (2.5%, w/v) were prepared in water and allowed to soak overnight at room temperature, and then transferred to microwave vessels containing a magnetic stir bar. Vessels containing insoluble fiber suspensions were then subjected to microwave radiation using the MARSXpress™ microwave system (CEM Corporation, Mathews, NC, USA) at 800 W,

180 °C for 30 min with 10 min of come-up[6] time under constant stirring at medium level. Samples were cooled to room temperature in a cold-water bath, centrifuged (8,000 g, 20 min, 15 °C), and 1 mL aliquots of supernatants were taken for measurement of total carbohydrate content. The total solids content of the treated IDF suspensions was reduced to 1% (w/v) by addition of water. Suspensions of microwave-treated IDFs were subjected to the enzyme hydrolysis C protocol described in Section 3.3.2. Briefly, suspensions were incubated with protease (P1236 Sigma, 0.1 U/g IDF, 50 °C for 4 h, and boiled for 15 min) followed by incubation with feruloyl esterase (E-FAEZCT Megazyme, 30 U/g IDF, 50 °C for 4 h, and boiled for 15 min). IDF suspensions were centrifuged (8,000 g, 20 min, 15 °C) and supernatants collected separately. Insoluble fibers were re-suspended in water (1% w/v) and incubated with carbohydrate hydrolyzing enzymes [Quinoa: endopolygalacturonase (E-PGALS Megazyme, 70 U/g IDF) + endo-1,4-β-galactanase (E-GALN Megazyme, 100 U/g IDF) + cellulase (C2605 Sigma, 70 U/g IDF); Pearl Millet: endo-1,4-β-xylanase (X2629 Sigma, 70 U/g IDF) + cellulase (C2605 Sigma, 70 U/g IDF)] at 50 °C for 24 h. Supernatants, which were collected separately, were added back to the IDF suspensions after incubation with carbohydrate hydrolyzing enzymes and freeze-dried. Freeze-dried samples were ground with a mortar and pestle to a coarse powder and sieved to collect a powder that had a particle size <500 μm. Due to the effects of microwave treatment and enzymatic hydrolysis on lignocellulosic-polysaccharide matrices, it was hypothesized that a portion of the fiber that remained insoluble after treatments became susceptible to microbial degradation and fermentation.

[6] Refers to the time in the program during which the microwave has to reach the pre-determined treatment temperature. This time is in addition to the 30 min at the pre-determined treatment temperature.

Thus, treatments of IDF generated fiber substrates that were constituted by 3 types of fiber: 1) soluble-fermentable (SFF), 2) insoluble-fermentable (IFF), and 3) insoluble-non-fermentable (INFF).

4.3.2 Determination of Total Carbohydrate Content on Supernatants of Treated Fiber Substrates

The amount of solubilized carbohydrate generated by treatments and enzymatic hydrolysis was quantified by the phenol-sulfuric acid method (Dubois et al., 1956). Aliquots (1 mL) of supernatants from microwave-treated and enzymatically-hydrolyzed IDF suspensions were diluted 100 times. Total carbohydrate content was measured in the diluted samples. Values for total carbohydrate content from microwave treatment were corrected for carbohydrate content in the suspensions of untreated fibers (see section 3.3.3, Chapter 3).

4.3.3 In Vitro Lower-gastrointestinal Fermentation of Treated Fiber Substrates

Lower-gastrointestinal fermentation of fiber substrates was simulated according to Lebet et al. (1998), with some modifications according to Rose et al. (2010b). Figure 4.1 describes how the different types of treated substrates were generated. Treated fiber substrates (MT=microwave-treated; M/ET=microwave + enzyme-treated), each containing a specific combination of the three types of fiber generated by treatments (SFF, IFF, and INFF) and total dietary fiber without treatment (TDF) from quinoa and pearl millet were weighed, in amounts equivalent to 40 mg of total carbohydrate, into anaerobic culture tubes (one tube/replicate, triplicates for each sampling period at 0, 6, 12, and 24 h). Anaerobic carbonate-phosphate buffer (Durand et al., 1988) was prepared and sterilized by autoclaving at 121°C for 1 h. Immediately after autoclaving, a constant

stream of carbon dioxide was bubbled through the buffer (to maintain anaerobiosis) and 0.25 mg/L of cysteine-hydrochloride was added as a reducing agent. After the buffer color changed, indicating reducing conditions, 4 ml were added to each tube along with 100 µl of Oxyrase (Oxyrase for broth, Oxyrase, Inc., Mansfield, OH, USA) to remove residual oxygen. After flushing the tube headspace with a stream of carbon dioxide, tubes were sealed anaerobically with a rubber stopper and stored at 4 °C overnight to allow for hydration of the fiber substrates. The next day, fecal samples were collected from three healthy individuals consuming their regular and unspecified diets and who had not been under antibiotic treatment in the last 3 months. Fecal samples were stored on ice and tightly sealed in plastic with air expelled immediately after collection and used within 2 h. Equal amounts of each fecal sample were combined and homogenized with 3 parts sterile anaerobic carbonate-phosphate buffer (prepared and maintained as described above), and then filtered through 4 layers of cheesecloth. Sealed anaerobic tubes containing hydrated fiber substrates were opened and 0.8 ml of fecal slurry was added to inoculate each tube under constant carbon dioxide flushing. The tubes were immediately re-sealed and incubated at 37 °C with gentle shaking. At each sampling period (0, 6, 12, 24 h), each tube set was removed from the water bath. A needle attached to a graduated syringe was inserted through the rubber stopper to measure gas production. The volume indicated by the displacement of the syringe plunger was recorded as the amount of gas produced. The tubes were then opened, and microbial activity was stopped by adding 0.4 ml of 2.75 mg/ml copper sulfate solution (containing 12.5 mg/ml of myo-inositol, as an internal standard for residual carbohydrate analysis). The pH of the fermented slurry was measured, and a 0.4 ml aliquot was combined with 0.1 ml of 5% phosphoric acid

(containing 50 mM 4-methyl valeric acid, as an internal standard for SCFA analysis), and mixed and frozen (-40°C) until ready for quantification of short chain fatty acids. The remainder of the fermented slurry was frozen separately for DNA extraction.

4.3.4 Quantification of Short Chain Fatty Acids

Short chain fatty acids were quantified from acidified fermentation slurries obtained from in vitro fermentation. Aliquots of fermentation slurries were thawed and then centrifuged at 13,000 rpm for 10 min. A 4 µl aliquot of supernatant was injected onto a HP 5890 GC equipped with a Nukol capillary column (30 m Å~ 0.25 mm ID, 0.25 µm bonded phase, Supelco, Bellefonte, PA) under the following conditions: injector temperature, 230 °C; detector temperature, 230 °C; detector, FID; initial oven temperature, 100 °C; temperature program, 8 °C/min to 192 °C, with hold for 3 min at final oven temperature; carrier gas, helium at 0.75 ml/min. Identification and flame ionization detector (FID) response factors for acetate, propionate, and butyrate relative to an internal standard (4-methyl valeric acid) were calculated by injecting a SCFA standard mix (Supelco, Bellefonte, PA, USA), and SCFA in samples were quantified by measuring the peak areas for acetate, propionate, and butyrate relative to 4-methyl valeric acid.

4.4 Results and Discussion

4.4.1 Processing of Quinoa and Pearl Millet Insoluble Dietary Fibers to Generate Treated Fiber Substrates

Suspensions of IDF and water (2.5%, w/v) were first subjected to microwave treatment at 180 °C for 30 min under constant stirring at medium level. Microwave-treated fiber substrates (MT-substrates) were then subjected to a sequential incubation with protease, feruloyl esterase, and carbohydrate-hydrolyzing enzymes (quinoa IDF: endo-

polygalacturonase + endo-β-1,4-galactanase + cellulase; pearl millet IDF: endo-β-1,4-xylanase + cellulase) to generate microwave + enzyme treated fiber substrates (M/ET-substrates). The enzymes in the sequential incubation were chosen to mimic an enzymatic deconstruction of plant cell wall material. Microwave-treatment and enzymatic hydrolysis of IDF suspensions generated treated fiber substrates with varying degrees of solubility and fermentability (Figure 4.2). According to the principle of microwave treatment, microwave radiation aids in the breakdown of the recalcitrant structure of lignocellulosic materials by creating "hot-spots" on the more polar and less crystalline sections of the matrix that expand or "explode" (Hu & Wen, 2008). Due to this effect, it was expected that microwave-treated insoluble fiber alone would have improved fermentability over the untreated insoluble fiber. Exposure of the insoluble lignocellulosic-polysaccharide matrix to microwave radiation would create pores to facilitate the attachment of bacterial cells and enzymatic hydrolysis for degradation of the insoluble material (Amrein et al., 2003; Taherzadeh & Karimi, 2008). Accordingly, the portion of fiber that remained insoluble after microwave treatment consisted both of a fraction with increased susceptibility to microbial degradations (fermentable) and a non-fermentable fraction that retained the recalcitrance of the insoluble dietary fiber. As previously described in Chapter 3, the soluble fiber fraction of the treated fiber substrates generated by microwave and enzyme treatments differed in composition and structure depending on the source of IDF.

For quantification of total soluble fiber content in quinoa and pearl millet treated substrates, the original soluble fiber found in the whole grain flours was added back to

the treated substrates in the same proportion found in total dietary fiber from whole grain (quinoa total dietary fiber = 23.0% soluble; pearl millet total dietary fiber = 15.0% soluble). Tables 4.1 and 4.2 show the amounts of IDF that was solubilized by each treatment as well as the amounts of IDF that remained insoluble in the treated substrates. Total amount of soluble fiber content in MT-substrates from quinoa IDF was 34.0% and, it increased to 48.0% in M/ET substrates. For treated fiber substrates from pearl millet (Table 4.2), total soluble fiber content was much lower at 8.0% in MT-substrates and 12.0% in M/ET-substrates. Addition of original soluble fiber increased soluble fiber content for quinoa to levels ranging from 46.0% to 57.0% for MT and M/ET-substrates, respectively. Treated fiber substrates from pearl millet had total amounts of soluble fiber that ranged from 20.0-23.0% after incorporation of original soluble fiber. As discussed in Chapter 3, pearl millet insoluble fiber was less susceptible to being solubilized by treatments than quinoa IDF, and increasing soluble fiber proved difficult. Perhaps a different type of hydrothermal treatment with harsher conditions may be required for these cell wall materials with comparably high level of lignification (pearl millet IDF = 17.0% lignin). As indicated by previous characterization of the fiber solubilized by treatments (Section 3.4.3, Chapter 3), the soluble fraction of treated substrates from quinoa consisted of fibers with predominantly linear structures mainly arising from glucans (cellulose and/or xyloglucan) and galacturonans (Table 3.13). On the other hand, soluble fiber fraction in the treated substrates from pearl millet had a higher degree of branching and mainly arose from arabinoxylans and a smaller portion came from cellulose (Table 3.14).

4.4.2 Fermentability of Treated Fiber Substrates

In addition to the soluble fiber fractions of the treated substrates, the structure of the remaining microwave-treated and microwave+enzyme-treated insoluble fiber had been postulated to be more susceptible to microbial degradation due to the weakening of the recalcitrant polymer matrix made up of lignin and partially crystalline cellulose in combination with hemicelluloses. Large improvements in fermentability were observed of insoluble fiber. Figure 4.2 shows the different amounts of each type of fermentable (and nonfermentable) fiber in the treated fiber substrates. The amounts of fermentable fiber were calculated based on the total SCFA produced after the 24-h in vitro fecal fermentation (Appendix B.1). Each treated fiber substrate is designated by its source and the total amount of fermentable fiber it contains (soluble fermentable + insoluble-fermentable). Total dietary fiber substrates without treatments (TDF) were ~50.0% fermentable (quinoa TDF= 49.0%, pearl millet TDF-51.0%) and MT-substrates only had a slight increase in total fermentable fiber (quinoa-MT=52.0%, pearl millet-MT=57.0%). This latter increase was mainly due to the solubilization effect of microwave treatment on quinoa. Since the INFF fraction remained virtually unchanged with just MT in substrates from both sources, it is assumed that the fiber that was solubilized arose from the IFF fraction, which significantly decreased in the case of quinoa. However, in the case of MT-substrate from pearl millet, both SFF and IFF increased slightly. M/ET-substrates had a substantially higher amount of total fermentable fiber in comparison to TDF and MT-substrates at ~80.0%. In comparison to MT-substrates, IFF in quinoa-M/ET increased from 6.0 to 14.0%, and in pearl millet M/ET the increase was much greater at 37.0 to 59.0%. Thus, although the amount of total fermentable fiber did not differ

significantly between quinoa and pearl millet treated substrates, the proportion of each type of fermentable fiber varied. In quinoa treated substrates, SFF was the predominant fraction, and IFF was the main fermentable fraction in the case of pearl millet treated substrates (Figure 4.2).

4.4.3 In Vitro Fecal Fermentation of Untreated Insoluble Dietary Fiber and Treated Fiber Substrate from Quinoa and Pearl Millet

4.4.3.1 Untreated Insoluble Dietary Fibers

Insoluble dietary fiber is generally poorly fermentable and not likely to have much effect on the colonic microbiota. Figure 4.3 shows fairly low initial gas and short chain fatty acid (SCFA) production during in vitro fecal fermentation of untreated total dietary fibers (TDF) from quinoa and pearl millet. The rates of gas production between quinoa and pearl millet differed considerably with pearl millet much lower in the first 6 h fermentation, though was as fermentable as quinoa by 12 h. No significant difference ($P < 0.05$) in gas production at 24 h was found between the two untreated TDF samples despite their differences in soluble fiber contents (23.0% quinoa TDF, 15.0% pearl millet TDF) and overall composition (galacturonans and xyloglucans in quinoa TDF, arabinoxylans in pearl millet TDF). As noted above, this was due to partial fermentation of the insoluble fraction. Based on their SCFA production, TDF substrates from pearl millet were estimated to contain higher amounts of IFF than quinoa (Figure 4.2). On the other hand, quinoa TDF is mainly composed of pectic polysaccharides that have higher water holding capacity, a property that has been reported that allows microorganisms to

penetrate or attach to the undigested material and to degrade it (Bourquin et al., 1996; Stephen & Cummings, 1979).

Similar to gas production, SCFA production began to plateau after 12 h of fermentation and by the end of the 24-h period. Untreated TDF substrates generated around 300 μmol of SCFA per 40 mg of carbohydrate, half of the amount generated by the soluble fast fermenting control, fructooligosaccharides (FOS). Compositional differences between total dietary fiber of quinoa and pearl millet TDF did not affect SCFA production rates. Although insoluble fibers ferment at a slower rate than soluble fibers, they generally produced appreciable amounts of SCFAs (McBurney & Thompson, 1990). Thus, in addition to promoting laxation, decreasing transit time, and binding substances such as bile acids and carcinogens, insoluble dietary fibers also exert physiological benefits in the colon via generation of SCFAs. The complex nature of insoluble dietary fibers may allow for the delivery of fermentation end products to more distal regions of the colon (McIntyre et al., 1993).

4.4.3.2 Improved Fermentability of Treated Fiber Substrates

As discussed in Section 4.4.2, the composition of the fermentable fiber in the treated substrates from quinoa consisted mainly of SFF fraction and in pearl millet treated substrates the predominant fraction was IFF. Gas and SCFA production data for MT-substrates is presented in Figure 4.4. As expected, MT-substrates from both grain sources generated higher amounts of gas than untreated TDF substrates. However, significant increases ($P<0.05$) in gas production were only discernible during 6 h and 12 h of fermentation. After 12 h, gas production plateaued and no significant differences

were found at 24 h. MT-substrates from pearl millet fiber had a slower initial rate of gas production than MT-substrates from quinoa fiber.

Significant increases in SCFA production were also found in comparison to the untreated TDF substrates. MT-substrates from both quinoa and pearl millet generated higher amounts of SCFAs than TDF substrates during the first 12 and 24 h of fermentation, although increases at 24 h were moderate. It is noteworthy that although the initial rate of gas production as slow for MT-substrates from pearl millet, their SCFA production was significant in this period. This indicates significant fermentation in the initial period, but low hydrogen gas production. According to Bernalier et al. (1999), the end products of fermentation depend on the type and availability of the carbohydrate substrates as well as the bacteria involved and their fermentative biochemical pathways. Thus, results from this study suggest that the composition of fermentable fiber in pearl millet MT-substrates, which was predominantly insoluble, supported the growth of bacterial groups that favored the production of SCFAs instead of gas during the first hours of fermentation.

In order to assess the fermentability of fiber that was solubilized by microwave treatment, a sample of the SFF fraction from MT-quinoa was used as a substrate for in vitro fecal fermentation. As is evident in Figure 4.5, Quinoa-SFF obtained from MT had an initial slow rate of gas production, then increased dramatically after 6 h and plateaued after 12 h of fermentation. Concurrently, SCFA production was fast in this period. This fermentation profile is interesting because the MT-substrate from quinoa that contained all three types of fibers (46.0% SFF, 6.0% IFF, and 48.0% INFF) had a rapid initial rate of gas production. This indicates that the relatively fast gas production in the initial

fermentation stages of MT-substrate from quinoa was not only due to the SFF fractions, but to the portions of IFF resulting from microwave treatment. An initially slow gas production rate is a desirable trait for a soluble fiber substrate, in particular, because soluble fibers are more functional in food systems and slow gas production would reduce abdominal discomfort that is associated with high gas producers such as FOS (Kaur et al., 2011). Quinoa-SFF substrate warrants further investigation to analyze what characteristics of the substrate determine this low gas, high SCFA fermentation profile.

Combining microwave-treatment with enzymatic hydrolysis, further solubilized the TDF substrates of quinoa. The microwave+enzyme treated (M/ET) substrate from quinoa contained 57.0% SFF; and for pearl millet SFF content was only 23.0% with the majority of its fermentable fiber insoluble (59.0% IFF). As shown in Figure 4.6, M/ET-substrates had a significant increase in gas and SCFA production compared to TDF and MT-substrates. M/ET-substrate from quinoa was fermented at a faster rate (tracked by gas production) than the M/ET-substrate from pearl millet, mainly because of its significantly higher SFF content. Although initial rates differed between quinoa and pearl millet, gas production reached comparable amounts after 12 h of fermentation as bacterial groups probably begin to adapt and degrade IFF in the M/ET-substrate from pearl millet. SCFA production of M/ET-substrates is presented in Figure 4.6 (B). Similar to results in gas production, M/ET-substrates had a comparably higher SCFA production. Regardless of botanical origin, amounts of total SCFAs generated over the entire 24-h fermentation period were comparable. These results further show that the fermentable property of insoluble fiber can be increased by these treatments. As estimations for fermentable fiber

contents indicated, significantly higher amounts of IFF in treated substrates from pearl millet compensated for their significantly lower amounts of soluble fiber than quinoa resulting in comparable fermentation profiles.

4.4.3.3 Short Chain Fatty Acid Profiles from In Vitro Fecal Fermentation of Treated Fiber Substrates

Besides generating higher amounts of SCFAs, treated fiber substrates obtained from quinoa and pearl millet also had an effect on the profile of the individual SCFAs (Table 4.3). For the blank fecal samples, the fecal microbiota used in the in vitro fecal fermentation study was predominantly propiogenic. As previously discussed, microwave treatment alone had a discernible effect on increasing SCFA production. There was an increasing acetate and decreasing propionate trend observed for all the treated fiber substrates. Butyrate proportion remained stable over the 24-h fermentation period with a slight decrease between 6 h to 12 h of fermentation in the blank samples. M/ET-substrates produced significantly higher amounts of butyrate than their untreated counterparts and the amounts were comparable to butyrate amounts generated by FOS after 12 h and 24 h of fermentation. A significant increase in butyrate levels generated in the last stages of fermentation indicates that modification of these TDFs generated substrates that can potentially deliver butyrate to more distal regions of the colon. Quinoa-SFF substrate produced high amounts of total SCFAs, though it had the lowest butyrate proportion out of all the samples.

As % soluble fiber content of the TDF and treated substrates increased, the increase in acetate and decrease in propionate proportions was more substantial. Interestingly, the

samples with highest proportions of propionate had lower degree of solubility. Propionate proportions correlated negatively with percent soluble fiber content of the fiber substrates (Table 4.4), suggesting that propiogenic bacterial groups may thrive with more insoluble fiber substrates. The significantly higher amount of total SCFAs produced by FOS was mainly due to the contribution of acetate, which constituted more than half of the total amount of SCFAs produced at each time point during the 24-h fermentation. There was a strong positive correlation between percent soluble fiber content and acetate proportion, and no relationship was found between % soluble fiber content and butyrate proportions.

4.5 Conclusions

The modification of insoluble dietary fibers by microwave-treatment and enzymatic hydrolysis generated treated fiber substrates with varying degrees of solubility and fermentability. Increases in gas and SCFA production as well as causing shifts in SCFA profiles indicated improved fermentation profiles due to treatments. Treated fiber substrates generated significantly higher amounts of butyrate than untreated TDFs and, at 12 h and 24 h of fermentation, butyrate levels generated by M/ET substrates were comparable to those generated by FOS. Fibers from quinoa and pearl millet were also more propiogenic than FOS. Compositional differences resulted in the varying amounts of SFF and IFF, which, in turn, determined fermentability. Treated fiber substrates from quinoa had SFF contents that ranged from 46.0% to 57.0% after addition of its original soluble dietary fiber and IFF contents between 6.0% and 14.0%. Although soluble fiber content of treated substrates from pearl millet was significantly lower (~20.0%), its fermentability was on par with that of fiber substrates from quinoa due to their high

contents of IFF that ranged from 37.0% to 59.0%. Therefore, improvements in fermentability were not due primarily to increases in fiber solubility, but by increase in a fermentable insoluble fiber material. The common approach to produce fermentable oligo- and polysaccharides from insoluble dietary fibers involves their complete solubilization, usually by chemical extraction methods. Results presented for insoluble fibers from pearl millet indicate that hydrothermal and enzymatic treatments can substantially increase fermentability without solubilizing the insoluble dietary fibers. The physical effect that hydrothermal treatments have on the configuration of the insoluble polysaccharide matrix in combination with enzymatic hydrolysis results in greater susceptibility to microbial degradation. An insoluble fiber that has been made more fermentable will have a slower fermentation profile and will be fermented at more distal regions of the colon. Unexpectedly, microwave solubilization of quinoa IDF produced a substrate with a fairly high fermentability characterized by low gas production during the initial stages of fermentation. Thus, quinoa-SFF constitutes a soluble fiber substrate with promising functional applications because its fermentation profile suggests that it can deliver significant amounts of SCFAs to proximal regions of the colon without causing abdominal discomfort due to excessive gas production.

Overall, this study showed that fermentability of insoluble dietary fibers can be increased without complete solubilization and their fermentation profiles manipulated to generate substrates that have initially slow rates of gas production and that can deliver SCFAs to distal regions of the colon. Further experimentation and analysis is postulated to lead to

the design of targeted methods of modification to create fermentable substrates with compositions and structure that will drive specific fermentable properties.

Table 4.1 Insoluble and soluble carbohydrate content of fiber substrates generated by microwave treatment at 180 °C and enzyme hydrolysis C[1] of quinoa insoluble dietary fiber (99.6% insoluble).

Quinoa IDF (%)				
Type of Fiber	MT[2]	MT + Original SDF	M/ET[3]	M/ET + Original SDF
Microwave-treated Insoluble[4]	**66**	**54**	**52**	**43**
Microwave-solubilized	34 ± 0.06	28 ± 0.03	34 ± 0.06	28 ± 0.03
Enzyme-solubilized	--	--	14 ± 0.05	11 ± 0.05
Original Soluble[5]	--	18	--	18
Total Soluble Fiber Content[6]	**34**	**46**	**48**	**57**

[1]See Chapter 3, Section 3.3.2
[2]MT= microwave treated insoluble dietary fiber
[3]M/ET= microwave + enzyme treated insoluble dietary fiber
[4]Amounts of microwave-treated insoluble are calculated by difference.
[5]Original soluble fiber is re-incorporated into the sample in the amounts found in total dietary fiber.
[6]Total amount (%) of soluble carbohydrate per substrate is equal to the sum of original, microwave-solubilized, and enzyme-solubilized fiber.
Values presented are the averages of duplicate runs of each treatment.

Table 4.2 Insoluble and soluble carbohydrate content of fiber substrates generated by microwave treatment at 180 °C and enzyme hydrolysis C[1] of pearl millet insoluble dietary fiber (99.9% insoluble).

Pearl Millet IDF (%)				
Type of Fiber	MT[2]	MT + Original SDF	M/ET[3]	M/ET + Original SDF
Microwave-treated Insoluble[4]	**92**	**80**	**88**	**77**
Microwave-solubilized	8 ± 0.03	7 ± 0.01	8 ± 0.06	7 ± 0.01
Enzyme-solubilized	--	--	4 ± 0.04	3 ± 0.04
Original Soluble[5]	--	13	--	13
Total Soluble Fiber Content[6]	**8**	**20**	**12**	**23**

[1]See Chapter 3, Section 3.3.2
[2]MT= microwave treated insoluble dietary fiber
[3]M/ET= microwave + enzyme treated insoluble dietary fiber
[4]Amounts of microwave-treated insoluble are calculated by difference.
[5]Original soluble fiber is re-incorporated into the sample in the amounts found in total dietary fiber.
[6]Total amount (%) of soluble carbohydrate per substrate is equal to the sum of original, microwave-solubilized, and enzyme-solubilized fiber.
Values presented are the averages of duplicate runs of each treatment.

Table 4.3 Short chain fatty acid ratios generated by TDF[1], MT, and M/ET fiber substrates from quinoa and pearl millet, Quinoa SFF-86F, FOS and Blank at 6, 12, and 24 h of in vitro fecal fermentation.

Fiber Substrate	**acetate : propionate : butyrate**			
	0 h	6 h	12 h	24 h
Blank	56:22:22	41:53:7	31:62:7	32:57:11
Quinoa TDF-49F[3]	--	49:37:14	41:43:16	41:42:17
Quinoa MT-52F	--	58:27:15	45:39:16	44:39:17
Quinoa M/ET-71F	--	61:22:17	49:33:17	48:34:18
Quinoa SFF-86F	--	68:20:12	56:30:13	56:30:14
PMillet[4] TDF-51F	--	47:34:19	42:42:16	43:40:17
PMillet MT-57F	--	57:27:16	44:39:17	45:38:17
PMillet M/ET-82F	--	58:23:19	51:32:17	52:31:18
FOS	--	76:10:14	61:21:18	59:24:17

[1]Abbreviations: TDF = untreated total dietary fibers; MT = microwave-treated fiber substrates; M/ET = microwave+enzyme-treated fiber substrates.
[2]Indicates % fermentability of each substrate (e.g. Quinoa TDF-49F = 49% of untreated total dietary fiber from quinoa sample is fermentable).
[3]PMillet = pearl millet

Table 4.4 Correlation coefficients indicating relationships between % soluble fiber content of TDF, MT, and M/ET substrates and proportions of acetate and propionate throughout the 24 h fermentation period

R	% Acetate			% Propionate		
	6 h	12 h	24 h	6 h	12 h	24 h
% Soluble Fiber Content	0.92	0.88	0.85	-0.8	-0.78	-0.77

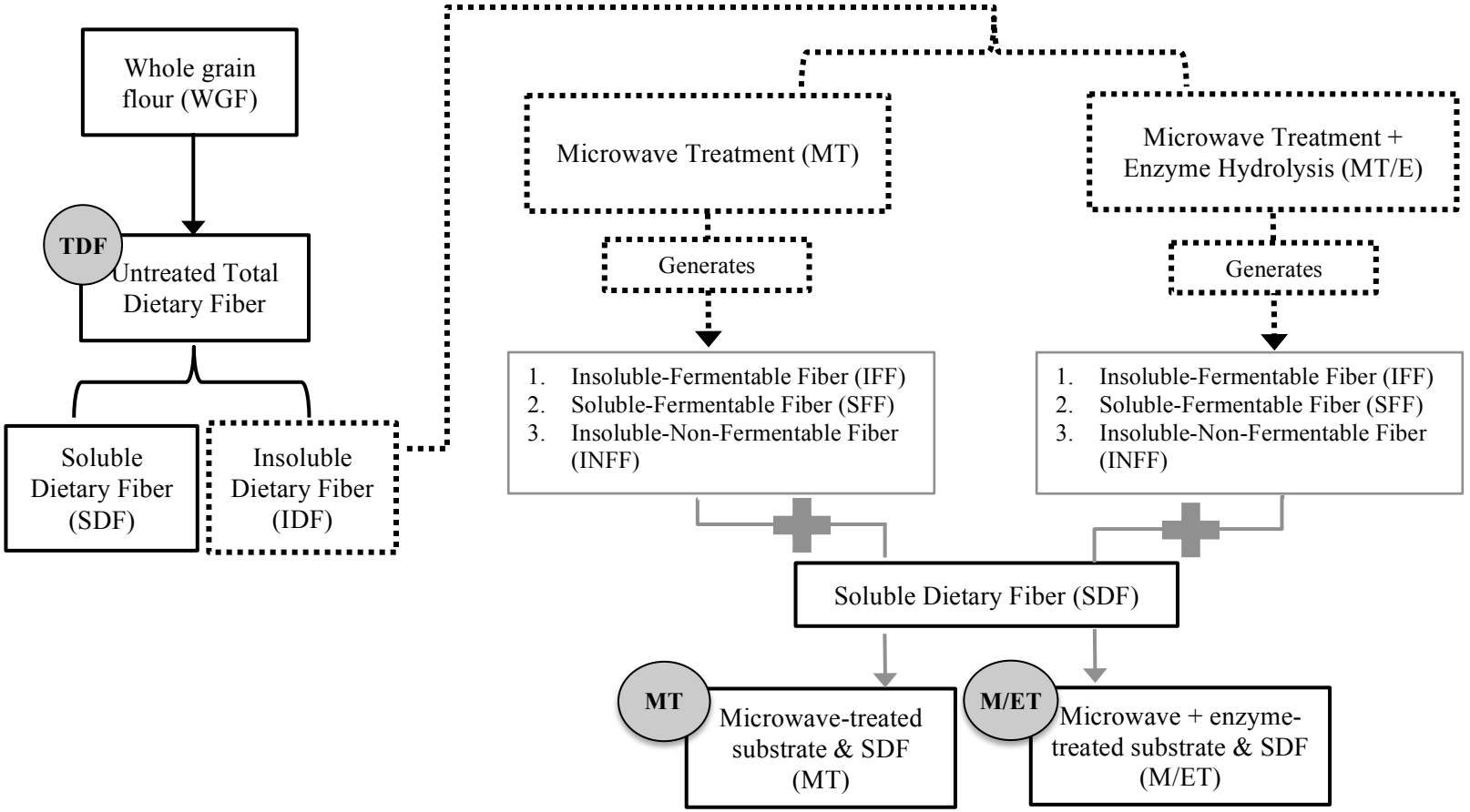


Figure 4.1 Generation of treated fiber substrates from insoluble dietary fibers isolated from quinoa and pearl millet grains. TDF refers to total dietary fiber without treatment, MT refers to microwave-treated insoluble fibers constituted by IFF, SFF and INFF plues the original soluble dietary fiber, and M/ET refers to microwave+enzyme treated insoluble fibers constituted by IFF, SFF and INFF plus the original soluble dietary fiber.

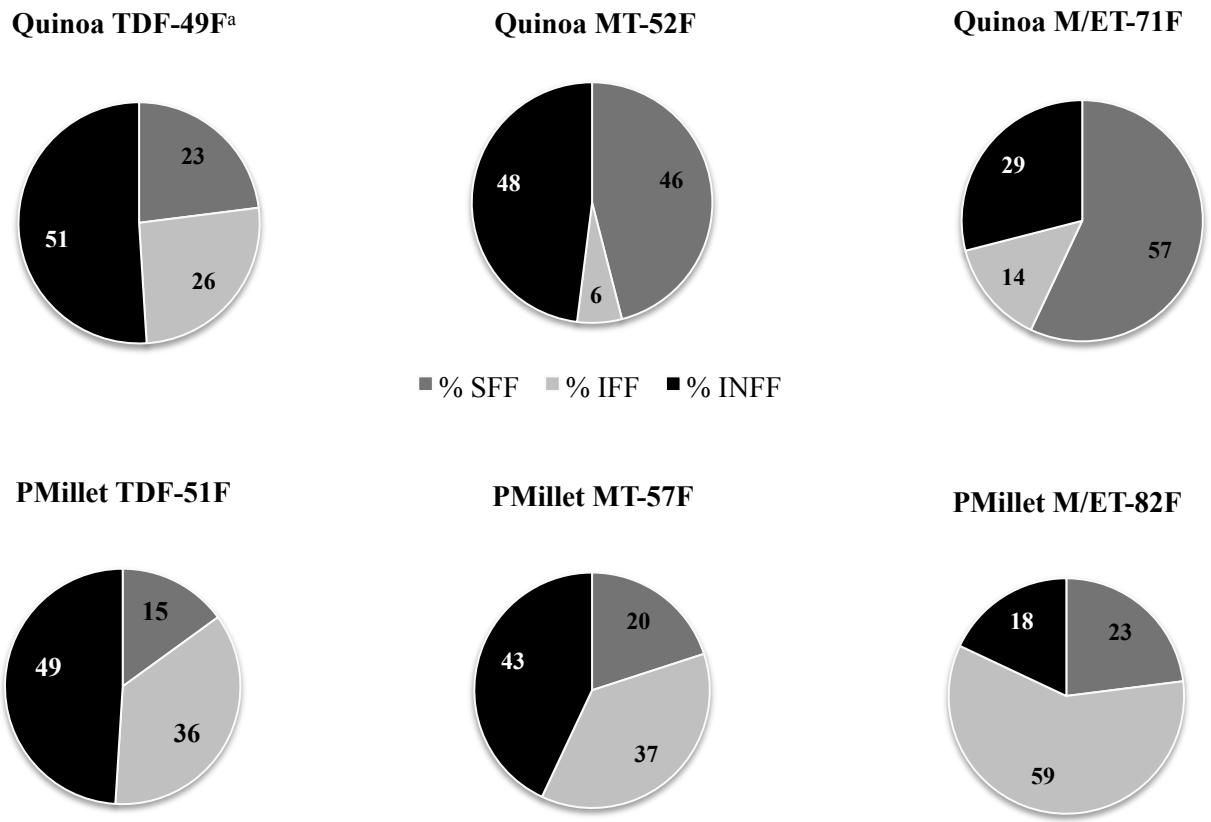
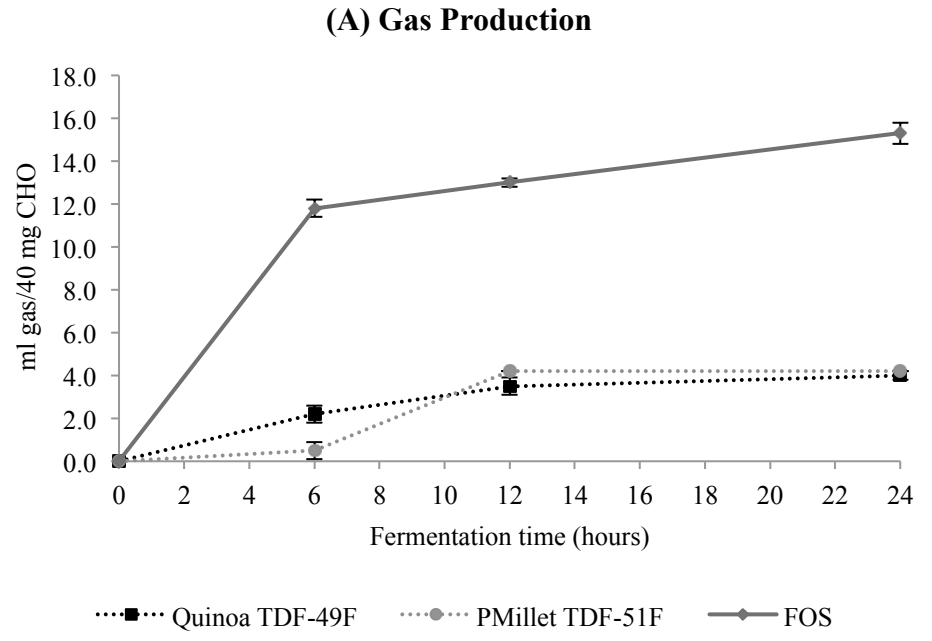


Figure 4.2 Soluble-fermentable fiber (SFF), insoluble-fermentable fiber (IFF), insoluble-non-fermentable fiber (INFF) contents (%) in TDF, MT, and M/ET substrates from quinoa and pearl millet. Fermentable fiber was calculated based solely on total SCFA production, amounts of gas and other fermentation end products were not accounted for in the calculations. [a]F refers to the total amount of fermentable fiber in the substrate (e.g. Quinoa TDF-49F = 49% fermentable substrate).



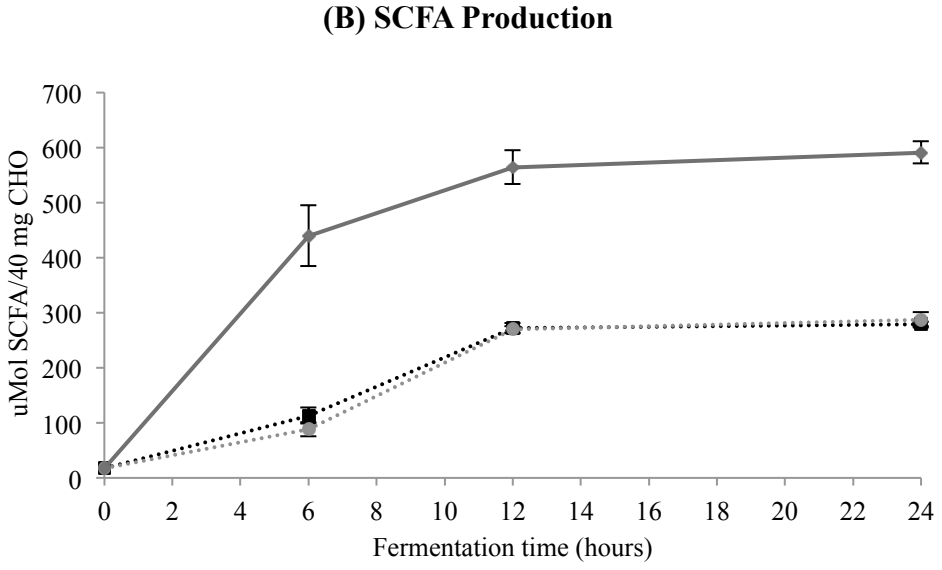


Figure 4.3 Gas (A) and short chain fatty acid (B) produced during in vitro fecal fermentation of untreated (TDF) substrates compared to the soluble fast-fermenting control FOS. Quinoa TDF-49F = untreated total dietary fiber from quinoa, 49% fermentable; PMillet TDF-51F = untreated total dietary fiber from pearl millet 51% fermentable. Blank has been subtracted from the data; error bars show standard deviation; some error bars are too small to see. Values are the average of triplicate measurements.

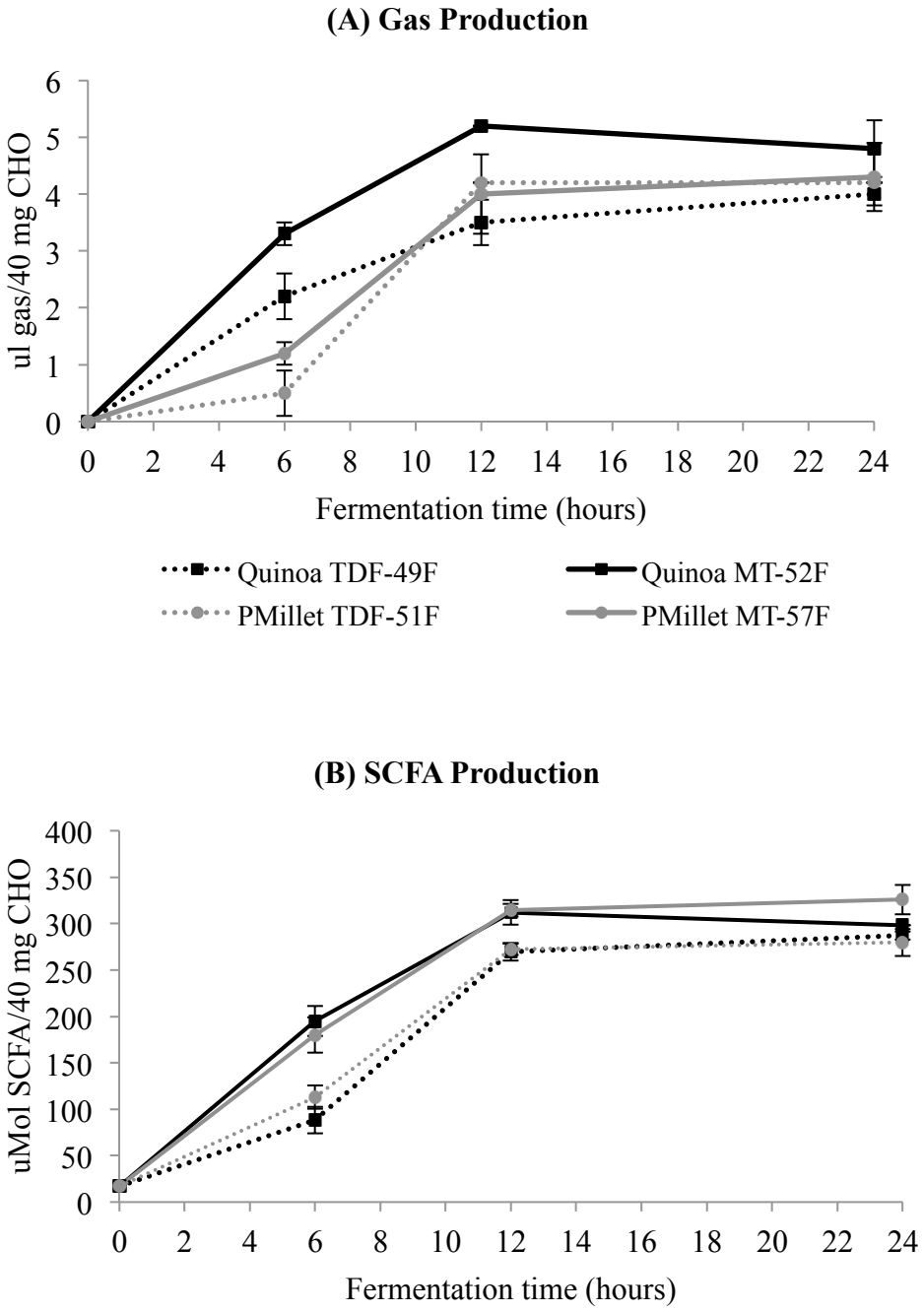


Figure 4.4 Gas (A) and short chain fatty acid (B) produced during in vitro fecal fermentation of untreated (TDF) and microwave-treated (MT) substrates. Quinoa TDF-49F = 49% fermentable; PMillet TDF-51F = 51% fermentable; 52F = 52% fermentable; 57F = 57% fermentable. Blank has been subtracted from the data; error bars show standard deviation; some error bars are too small to see. Values are the average of triplicate measurements.

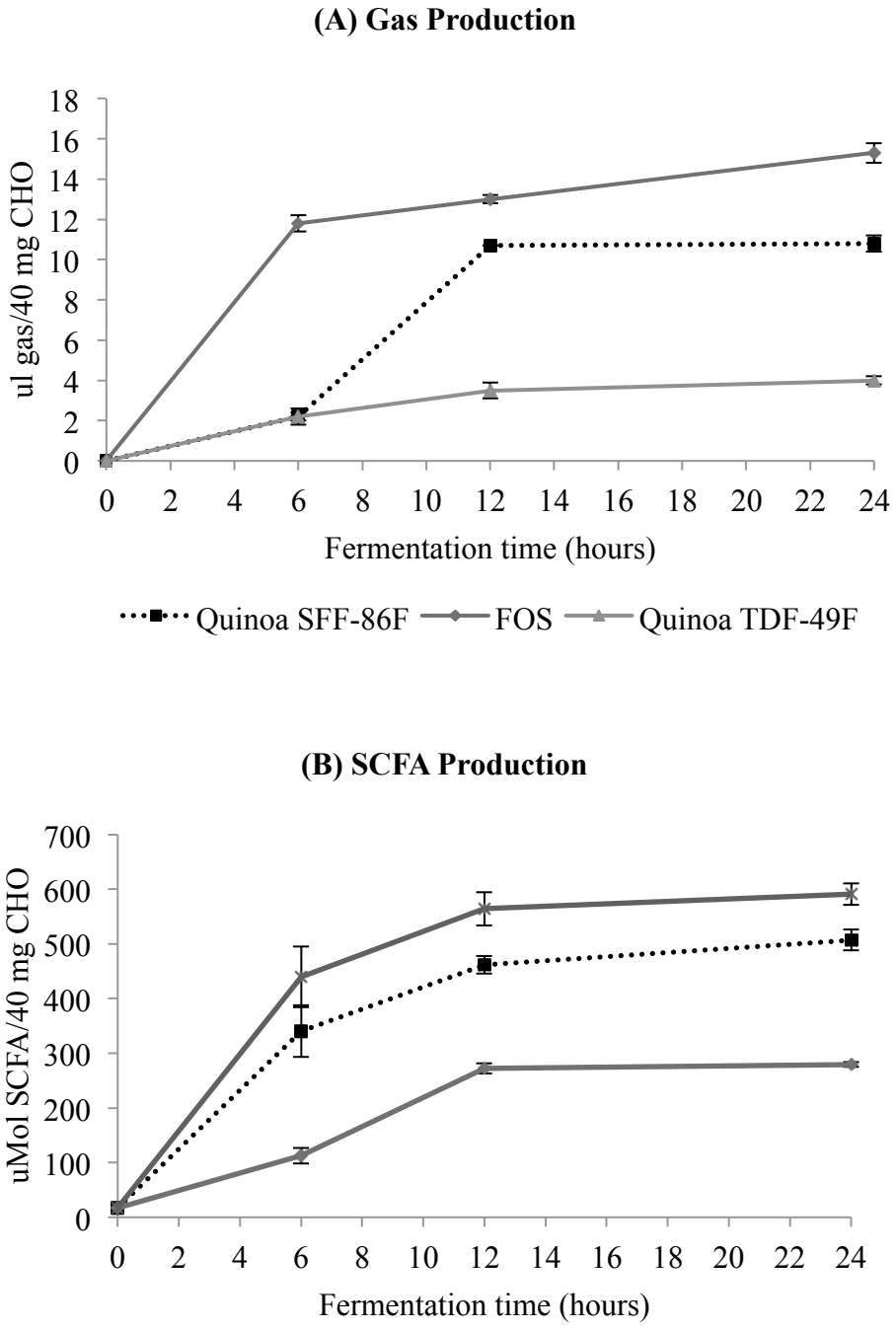


Figure 4.5 Gas (A) and short chain fatty acid (B) produced during in vitro fecal fermentation of soluble fermentable fiber from quinoa (Quinoa SFF) generated by microwave treatment compared to soluble fast-fermenting control FOS and Quinoa TDF-49F. Quinoa SFF-86F = 86% fermentable; Quinoa TDF-49F = 49% fermentable Blank has been subtracted from the data; error bars show standard deviation; some error bars are too small to see. Values are the average of triplicate measurements.

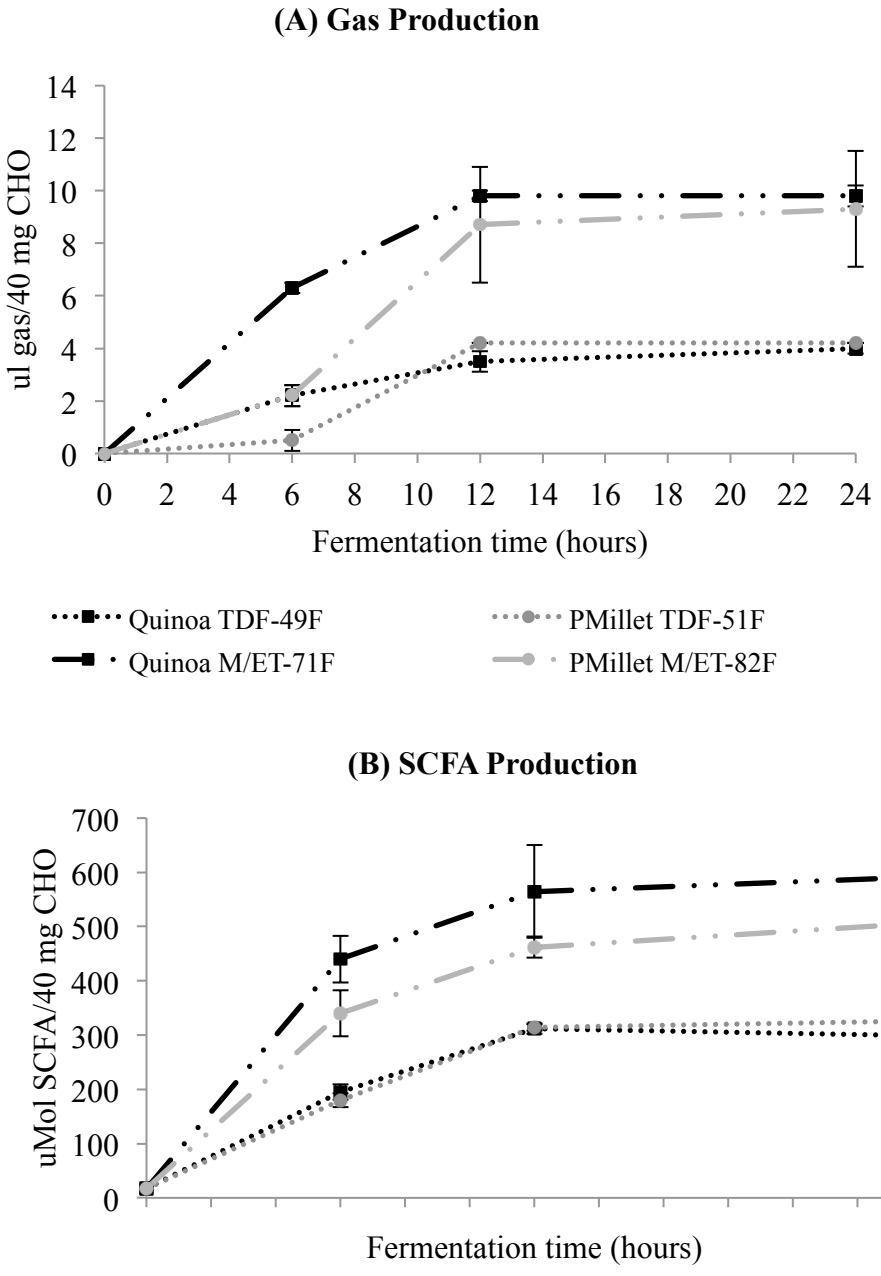


Figure 4.6 Gas (A) and short chain fatty acid (B) produced during in vitro fecal fermentation of microwave+enzyme-treated (M/ET) substrates from quinoa and pearl millet compared to TDF substrates from each grain. Quinoa M/ET-71F = 71% fermentable; PMillet M/ET-82F = 82% fermentable. Blank has been subtracted from the data; error bars show standard deviation; some error bars are too small to see. Values are the average of triplicate measurements.

CHAPTER 5. IN VITRO FECAL FERMENTATION EFFECT ON MICROBIAL COMMUNITIES OF TREATED QUINOA AND PEARL MILLET FIBER SUBSTRATES THAT VARY IN DEGREE OF FERMENTABILITY

5.1 Abstract

Soluble-fermentable (SFF), insoluble-fermentable (IFF), and insoluble-nonfermentable fiber (INFF) in varying amounts were generated from insoluble dietary fibers from quinoa and pearl millet treated with microwave-radiation and/or enzymatic hydrolysis and were subjected to in vitro fecal fermentation. Treatment of the insoluble dietary fibers resulted in their improved fermentability (i.e. increased total SCFA production, and increased butyrate and propionate proportions). In addition, the newly fermentable fiber substrates caused significant shifts in fecal microbial communities. The relative abundance of bacterial families changed according to type of fiber and time of fermentation. More specifically, fiber substrates derived from quinoa insoluble dietary fiber promoted the Ruminococcaceae family better than substrates derived from pearl millet or FOS, but pearl millet substrates were more bifidogenic than those from quinoa. Treated fiber substrates cause shifts in the fecal bacterial community that resembled the changes effected by fermentation with FOS. The combination of soluble-fermentable substrates with insoluble-fermentable carbohydrate polymers supported the growth of a larger of number of bacterial groups than the simple, readily fermentable FOS. Shifts in bacterial populations of the fecal microbiota suggest that the specific changes depend on

the type of fiber substrate and that mixtures of substrates might be prepared targeting specific dysbiotic conditions.

5.2 Introduction

Awareness of the importance of the gut microbiota as a factor that influences human health is growing. More specifically, that a wide-range of non-infectious gastrointestinal diseases such as inflammatory bowel disease, colon cancer, and irritable bowel syndrome have been associated to the metabolic activities of dysbiotic colonic microbiota (Nicholson et al., 2005; Rastall et al., 2005). It has also been shown that conditions such as metabolic endotoxemia, low-grade inflammation, glucose intolerance, which are risk factors associated with the onset of obesity and diabetes, are further linked to disorders of the gut microbiota (Cani et al., 2008; Musso et al., 2011; Zhang et al., 2010a). However, determining specific causative relationships is difficult as these diseases are multifactorial as well as phenotypically and/or genetically heterogeneous.

Although many of the gut species remain to be discovered, recent advances in analytical techniques have resulted in increased knowledge of the gut microbiota's diversity and functionality. Diet has been shown to be a major factor in shaping the composition of the gut microbiota (Faith et al., 2011; Turnbaugh et al., 2009; Zhang et al., 2012); and plant polysaccharides are a major source of fermentable substrate that reach the large intestine (Koropatkin et al., 2012). Many studies have shown that consumption of a variety of fermentable carbohydrates can improve the health status of the individual by resulting in modulation of host gene expression and metabolism (Delzenne et al., 2013; Tremaroli & Bäckhed, 2012). However, the approaches currently used for evaluation of health-

benefitting properties of fermentable carbohydrates do not account for factors such as the significant variation of gut microbiota composition between individuals (Benson et al., 2010; Ley et al., 2006) or the differentiation between healthy and dysbiotic microbiota (Sonnenburg & Sonnenburg, 2014). Furthermore, health-benefitting properties attributed to substrates deemed prebiotic are based on the "desirable" vs. "undesirable" classification of bacterial groups, however the parameters used for these classifications remain to be completely understood.

Despite the many uncertainties, the influence of fermentable carbohydrates on gut microbiota composition is clear, and is consistently observed both in vivo and in vitro. The identification and/or design of novel fermentable substrates for specific modulation of the gut microbiota is a reasonable goal, and can be thought of as a tool to improve health or eventually even to treat gut microbiota-associated diseases. In-depth studies have already shed light on the metabolic activity of bacterial groups and knowledge advances in the field have progressed many steps closer to an understanding of the effects that fermentable carbohydrate substrates have on the microbiota (Flint et al., 2008; Martens et al., 2009; Sela et al., 2008; Sonnenburg et al., 2005). From the food carbohydrate scientist's point of view, identification of carbohydrate substrates that effect positive changes in microbiota composition, the evaluation of the substrates' characteristics that cause those changes, and their relationship with health-benefitting fermentation end-products are some of the important targets. In this study, a variety of complex polysaccharide substrates produced by treatments of quinoa and pearl millet insoluble fiber to vary in composition, structure, solubility, and overall fermentability

were used in a 24-hour in vitro fecal fermentation to evaluate their effects on fecal microbial community composition

5.3 Materials and Methods

5.3.1 Microbiota Analysis

Genomic DNA from fecal samples at 0, 6, 12, and 24 hours of in vitro fermentation with fiber substrates made more fermentable was extracted using the FastDNA® Spin Kit for soil (MP Biomedicals, Ohio, USA) as per manufacturer's instructions with minor modifications. Briefly, 300 µl of fecal slurry were used. The concentration of extracted DNA was measured using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific Inc., Pittsburgh, PA, USA).

Microbial community profiling of the fecal samples was done using Illumina genomic sequencing. DNA samples were sent to the DNA Services Facility at University of Illinois at Chicago, where samples were amplified, sequenced, and data analysis was done. DNA was amplified using a dual PCR strategy that employs two stages of PCR amplification: (1) amplification of genomic DNA with a set of universal primers that contain common sequences positioned at the 5' end of the primer molecules. The primer set used includes a forward primer with Common Sequence 1 (CS1) named 515F (CS1_515F: 5'- ACACTGACGACATGGTTCTACAGTGCCAGCMGCCGCGGTAA) and a barcoded reverse primer with Common Sequence 2 (CS2) named 806R (CS2_806R: 5'- TACGGTAGCAGAGACTTGGTCTGGACTACHVGGGTWTCTAAT) (Caporaso et al., 2012). (2) The amplicons generated are briefly amplified with a second primer set that contains sequencing adapters for Illumina, sample specific barcodes and the common

sequences. PCR reactions for stage 1 were performed in 20 µL reaction volumes in 2X AccuPrime SuperMix II (Life Technologies # 12341-012) containing the desalted primers added at a final concentration of 0.5 µM. The initial denaturation step was at 95 °C for 5 min, followed by 28 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 45 s and elongation at 68 °C for 30 s, followed by a final elongation step at 68 °C for 7 min. PCR reactions for stage 2 were performed in 10 µL of the same 2X AccuPrime SuperMix II used in stage 1 (5µL of 2X SuperMix II, 2 µL water, 2 uL of barcoded primers, 1 µL of stage 1 PCR product). The initial denaturation step for stage 2 was at 95 °C for 5 min, followed by 8 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and elongation at 68 °C for 30 s, followed by a final elongation step at 68 °C for 7 min. PCR products and controls were verified by agarose gel electrophoresis and Qubit DNA concentration analysis. PCR products were pooled together and purified using AMPure XP Bead cleanup (0.6X buffer) followed by dilution to an appropriate concentration for Illumina sequencing. Sequencing data analysis was performed by Dr. Stefan Green at the DNA Services Facility at University of Illinois at Chicago. Statistical analyses for the description of diversity, analysis of similarity (ANOSIM)[7] to determine significant differences in microbiota composition between fiber substrates, and similarity percentages (SIMPER)[8] to identify the taxonomic groups that contributed to the differences were performed.

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[7] ANOSIM analyses are performed to determine if the sample groups are significantly different from each other. An "R-statistic" is generated, along with a p-value using a permutation approach.

[8] SIMPER identifies those taxa that are contributing most to each group. In addition, the SIMPER does pairwise comparisons of each two groups to determine which taxa are contributing the most to the differences between the compared groups.

5.4 Results and Discussion

5.4.1 Composition of Fecal Microbiota Community (FMC) Before In Vitro Fermentation of Treated Fiber Substrates

Genomic analysis was performed on an aliquot of the pooled fecal sample prior to in vitro fermentation to determine the composition of the original microbial community (Table 5.1). Results revealed that around 92% of the sequences detected belonged to three of the four most populated bacterial phyla in the human gut microbiota, namely, Firmicutes, Bacteroidetes and Actinobacteria (Qin et al., 2010). In accordance with estimates of Firmicutes abundance of this phyla in human gut microbiota (Jalanka-Tuovinen et al., 2011; Turnbaugh et al., 2008), this phylum predominated in the original fecal microbial community. Lachnospiraceae and Ruminococcaceae, which are the most abundant families in gut environments that belong to the Firmicutes phylum (Tap et al., 2009), constituted ~58% of the total sequences detected. The Bacteroidetes phylum (37% of sequences detected) was the second most abundant phylum present and 5% of the sequences detected corresponded to the Actinobacteria phylum. The Proteobacteria phylum, which includes a variety of pathogenic species and whose outer membranes are mainly composed of lipopolysaccharides (Gupta, 2000), was present in very low amounts constituting only 0.5% of the sequences detected.

5.4.2 Effects of In Vitro Fecal Fermentation of Treated Quinoa and Pearl Millet Fiber Substrates on α-Diversity of Microbial Communities

For in vitro fecal fermentation, fecal subsamples were incubated with treated insoluble fiber substrates from quinoa and pearl millet that had been subjected to microwave

treatment and enzyme hydrolysis to create carbohydrate substrates with varying degrees of fermentability (see Ch. 4, Table 4.3). It was previously shown in Chapter 2 that quinoa and pearl millet differ in composition and structure of its dietary fibers. Genomic DNA extracted from fecal samples after incubation with the treated insoluble fiber substrates for 6, 12, and 24 h was sequenced to analyze the changes in composition of the fecal microbial community as a response to the different fiber substrates.

Analysis of the compositional dissimilarity between microbial communities after in vitro fecal fermentation is presented in Figure 5.1. The Bray-Curtis dissimilarity test showed that the replicates per fiber substrate grouped well and that fiber substrate and time had definitive effects on microbial community composition. Microbial communities fermented with untreated and microwave-treated substrates clustered together. Microwave+enzyme treated substrates caused shifts in the microbial communities; fermentation of pearl millet substrates at all three time points and quinoa substrates at 24 h grouped with microbial communities that fermented FOS for 12 h. Microwave-solubilized fiber from quinoa resulted in the most divergent microbial communities at all three time points of fermentation. α-Diversity refers to the diversity within a specific site, community or habitat, and is often estimated by the number of species detected in a community (Lozupone & Knight, 2008). The Margalef's species richness and Shannon diversity index are descriptive of the α-diversity of the fecal microbiota communities (FMC). The Margalef's species richness is a measure of the total number of species detected in a community. As shown in Figure 5.2, richness of FMCs did not change dramatically after in vitro fecal fermentation with the treated fiber substrates. However,

the fermentation of FOS resulted in lower species richness compared to the control-no fiber (NF) and microwave-treated (Quinoa MT-52F) and microwave+enzyme-treated (Quinoa M/ET-71F) samples from quinoa fiber. The Shannon diversity index, which takes into account the abundance of the phylogenetic groups detected within a community, decreased significantly from the NF sample only in the case of in vitro fecal fermentation with the microwave-solubilized fiber from quinoa (Quinoa SFF-86F). Although, diversity for FOS and substrates from pearl millet (untreated = PMillet TDF-51F, microwave-treated = PMillet MT-57F, and microwave+enzyme-treated = PMillet M/ET-82F) was statistically equal, diversity values for FOS were significantly lower than diversity of the NF and quinoa substrate samples. The Quinoa SFF-86F resulted in decreased diversity of the microbial community compared to the rest of the fiber substrates including FOS. The Quinoa SFF-86F substrate predominantly consisted of unbranched glucan, galacturonan and arabinan oligosaccharides (see Chapter 3, Table 3.1). Similarly, FOS consists of linear fructan oligosaccharides often with one external or internal glucose moiety (Waterhouse & Chatterton, 1993). Studies have shown that members of the gut microbiota have different responses according to variations in structure, degree of polymerization, and molecular weight of oligosaccharides (Grootaert et al., 2009; Holck et al., 2011; Mandalari et al., 2007; Manderson et al., 2005; Van Craeyveld et al., 2008). The reduced diversity of the fecal microbiota samples for FOS and Quinoa SFF-86F is most likely due to their support of specific bacterial groups that thrive on simple polysaccharide structures.

5.4.3 Changes in Microbiota Composition After In Vitro Fecal Fermentation of Treated Quinoa and Pearl Millet Substrates

Although FMCs did not change substantially in terms of richness and diversity, significant differences in microbiota composition according to composite fiber substrate were observed. Analysis of similarity (ANOSIM) was used to test the significance of the differences between the samples and an R-statistic determined the magnitude of the difference between them (Table 5.2). As previously mentioned, analysis of compositional differences in these fecal microbiota communities are presented at the family[9] and species taxonomic levels. The bacterial families that contributed ~85% of the differences found between FMCs were Bacteroidaceae (Phyla: Bacteroidetes), Lachnospiraceae (Phyla: Firmicutes), Ruminococcaceae (Phyla: Firmicutes) and Bifidobacteriaceae (Phyla: Actinobacteria). Results of the ANOSIM showed that the composition of FMCs obtained after incubation with untreated (TDF) fiber substrates were not significantly different from those incubated with microwave-treated (MT) fiber substrates (Quinoa MT-52F, PMillet MT-57F). Although the overall composition of the FMCs did not differ significantly between untreated (Quinoa TDF-49F, PMillet TDF-51F) and microwave-treated (Quinoa MT-52F, PMillet MT-57F) substrates, gas and SCFA production did (see Chapter 4). The increase in gas and SCFA production from microwave-treated fiber substrates was due to an increase in the abundance of the microbiota as a response to a higher amount of fermentable carbohydrate. Pairwise comparisons of FMCs that were found to be significantly different are presented in Table

[9] The Family taxonomic level is used to present the data when the available data is not robust at the genus and/or species levels. Despite the advanced sequencing technology used here, many of the detected sequences cannot be assigned to genus or species taxons with certainty and remain categorized at the family level.

5.2. In general, results showed that differences in FMC composition were driven by differences in composition, structure, and fermentability of the substrates.

Figure 5.3 shows the average abundance (# of sequences) of the four key differentiating families in FMCs after incubation with untreated fibers from both grains (Quinoa TDF-49F and PMillet TDF-51F) and FOS. The main differences in composition between communities incubated with quinoa and pearl millet untreated fibers are discernible in the abundances of Ruminococcaceae and Bifidobacteriaceae families (3000 seq versus 2100 seq and 1000 seq versus 1800 seq, respectively). Statistical analysis using a parametric test (ANOVA) indicates that all four families differentiate the FOS bacterial community from both untreated quinoa and pearl millet bacterial communities. Lachnospiraceae and Bifidobacteriaceae were significantly more abundant in the FOS bacterial community than in quinoa and pearl millet-untreated fiber communities. Differences between FMCs from untreated fibers, which are predominantly insoluble (77% quinoa TDF and 85% pearl millet TDF), and FOS provide more proof that the different fiber substrates promote different bacterial groups causing changes in the composition of FMCs. The Ruminococcaceae family was favored by Quinoa TDF-49F to a significantly greater extent than by PMillet TDF-51F which, in turn, was more bifidogenic than quinoa. Members of the *Ruminococcus spp.,* a predominant member of the Ruminococcaceae family in the FMCs, are capable of hydrolyzing complex carbohydrates and acetate is the major end product of fermentation. The Ruminococcus species has been categorized as cellulolytic (Wedekind et al., 1988) that carries sophisticated enzyme systems on their cell surface which allow them to bind and break down cellulose (Schwarz, 2001). This

species is equipped to degrade glucan polymers with β-1,4-linkages, which are present in higher amounts in fiber substrates from quinoa because of its xyloglucan and cellulose contents. Thus, the significant increase in Ruminococcaceae family from quinoa fibers is consistent with the compositional characteristics of those substrates.

Another important finding is depicted in Figure 5.4. Increase in amount of soluble-fermentable and insoluble-fermentable fibers generated by the microwave+enzyme treatment significantly changed bacterial families during the in vitro fecal fermentation. The most evident changes were a significant decrease in Bacteroidaceae and significant increase in Lachnospiraceae observed for microwave+enzyme treated fibers (Quinoa M/ET-71F & PMillet M/ET-82F). In addition, the microwave+enzyme treatment combination resulted in significant increases in the abundance of Bifidobacteriaceae for substrates from both sources. Dominant members of the Lachnospiraceae family found in the FMCs, *Blautia spp., Coprococcus spp.* and *Roseburia faecis,* are butyrate producers (Barcenilla et al., 2000; Duncan et al., 2002a). Thus, a significant increase in the abundance of this bacterial family resulting from the in vitro fermentation of microwave+enzyme treated substrates may help explain the higher amount of butyrate that they generated (see Chapter 4). Studies have reported that the gut microbiota of IBD, obesity and NASH (nonalcoholic-steatohepatitis) patients are depleted of these Lachnospiraceae species (Frank et al., 2007; Spor et al., 2011; Zhu et al., 2013).

Figure 5.5 shows how the average abundances of the four major families in the FMCs from Quinoa M/ET-71F and PMillet M/ET-82F compare to the FMC from FOS. According to ANOSIM (Table 5.2), FMCs from FOS and PMillet M/ET-82F were not

significantly different from each other. Significant differences between the two substrates were only found in Bifidobacteriaceae, which was higher for FOS. The bifidogenicity of FOS and inulin-derived substrates has been reported in many studies (Bouhnik et al., 2004; Kolida et al., 2002; Rossi et al., 2005). Bifidobacterium species have oligosaccharide-degrading enzyme clusters that enable them to preferentially metabolize oligosaccharides (Bottacini et al., 2010; Pokusaeva et al., 2011). However, they are also able to feed on carbon sources other than fructans, a variety of oligosaccharides from arabinoxylans also have been shown to exert a bifidogenic effect as well (Grootaert et al., 2006; Hughes et al., 2007; Neyrinck et al., 2012). Some Bifidobacterium species are able to transport arabinoxylan-derived oligosaccharides into the cell and then degrade them intracellularly to monosaccharides (Gilad et al., 2010). The dominant members of the Bifidobacteriaceae in these FMCs were *Bifidobacterium breve, B. adolescentis* and *B. bifidus* (Figure 5.6 A) and the main effects according to different substrates were observed for *B. breve* whose abundance was only maintained by PMillet TDF-51F, and significantly increased by FOS and reduced by the other substrates. Interestingly, it has been previously reported that *B. breve* is not able to ferment arabinoxylo-oligosaccharides (Van Den Broek & Voragen, 2008). Further modification of pearl millet insoluble fiber, to contain higher amounts of soluble oligosaccharides and insoluble-fermentable fiber, may promote increases in Bifidobacteriaceae to abundances in the range found for FOS.

Different from substrates from pearl millet and FOS, fiber substrates from quinoa, Quinoa TDF-49F and Quinoa M/ET-71F, promoted the Ruminococcaceae family. An

important member of the Ruminococcaceae family, *Faecalibacterium prausnitzii,* was the predominant species of that family in the initial FMC and is one of the most abundant butyrate-producing bacterium in the gut microbiome of healthy individuals (Miquel et al., 2013). This species is of interest as it has been shown to be depleted in the microbiota of Crohn's disease patients (Fujimoto et al., 2013) and to have anti-inflammatory effects on cellular and TNBS colitis models (Sokol et al., 2008). In this study, FOS and all the treated fiber substrates significantly decreased the abundance of this bacterium (Figure 5.7 B). Although it has been shown that *Faecalibacterium prausnitzii* can metabolize a wide range of carbohydrate substrates, it has strict requirements for acetate in its growth medium and is extremely sensitive to oxygen (Duncan et al., 2002a; Duncan et al., 2002b); these factors, which cannot be precisely controlled in an in vitro fermentation setting, could have hindered the its growth. In addition, the survival and proliferation of a bacterium within a gut microbiota community that is undergoing changes in substrate availability depends on its ability to use the substrates and how fast it is able to adapt to the rapid changes.

In general, fermentation of the different treated fiber substrates resulted in significant shifts in bacterial groups within the fecal microbiota community. Furthermore, microwave and enzymatic treatments of insoluble dietary fibers from both grains increased the amount of fermentable fiber that specifically promoted the growth of bacterial groups that were also promoted by FOS most likely due to the availability of oligosaccharides of smaller size that may be preferred by some members of the Lachnospiraceae and Bifidobacteriaceae families.

The fermentation of Quinoa SFF-86F caused the greatest change in the FMC composition (Figure 5.8) and ANOSIM results confirmed that the FMC composition that resulted from Quinoa SFF-86F fermentation differed significantly from all the others (Table 5.2). Analysis of the sequences detected in the FMC revealed that Quinoa SFF-86F significantly favored the growth of Bacteroidaceae family, and *Bacteroides spp.* constituting 54% of the sequences detected for that family. Figure 5.9 shows the changes in family abundances that occurred over the 24-hour fermentation period. There is a clear preference of Bacteroides spp. for this substrate. The increase in Bacteroidaceae occurs at the expense of the other families, especially the Firmicutes. Predominant species from other families such as, *Coprococcus spp*., *Roseburia faecis*, and *Bifidobacterium breve* were significantly reduced.

5.4.4 Changes in Microbiota Composition Over 24-h In Vitro Fecal Fermentation of Treated Quinoa and Pearl Millet Fiber Substrates

Time was also a determining factor in the composition of FMCs providing further proof of the dynamic nature of that ecosystem. The number of families detected increased with time in all the treated fiber substrates from quinoa and pearl millet. Figure 5.10 shows the changes in average abundance of the dominant families at 0, 6, 12, and 24 h of fermentation of Quinoa TDF-49F and Quinoa M/ET-71F substrates. Bacteroidaceae increased considerably after 6 h of fermentation with Quinoa TDF-49F, but the opposite was the case for Quinoa M/ET-71F fiber in which the main increase was seen in Lachnospiraceae. After 12 h of fermentation, bacterial groups that were not detected in the initial microbial community, begin to proliferate. It is possible that as the

predominant bacterial groups degrade the substrates available, intermediate products of fermentation or fiber hydrolyzates become available and are used by the other bacterial groups originally present in smaller and undetectable proportions. In the case of fiber substrates from pearl millet (Figure 5.11), shifts in bacterial groups over the 24-h period of fermentation were slightly less distinguishable, mainly for the PMillet TDF-51F fiber sample in which diversity did not change dramatically. Although only one new family proliferated after 24 h of fermentation of PMillet M/ET-82F, the relative abundance of each family in the fecal microbial community changed considerably over time. Lachnospiraceae and Bifidobacteriaceae were favored between the 6 and 12 h time points, however, by the end of the fermentation period, Bacteroidaceae abundance returned to initial levels and Veillonellaceae appeared in minor amounts.

It has been described in the previous chapters that these fiber substrates arise from the insoluble dietary fibers which are fibrous composites of many types of polymers including carbohydrates, lignin, waxes and proteins (MacDougall & Selvendran, 2001; Selvendran, 1984). Both fermentable soluble and fermentable insoluble substrates are generated as a result of microwave radiation and enzymatic treatments. Due to the complexity of these fiber substrates, gut bacteria rely on multi-enzyme systems in order to utilize them. Bacterial groups in the gut differ in their preferences for carbohydrate utilization due to their linkage-specific degradative enzymes and when carbohydrate substrates are trapped in a lignocellulosic matrix, the utilization of those substrates is further complicated (Koropatkin et al., 2012). The shifts in microbial groups of fecal communities presented here suggest that treated fiber substrates comprised of soluble-

fermentable and insoluble-fermentable fibers that vary in composition, solubility, and polymer configuration, might create a sort of substrate gradient in which primary degraders or bacterial groups that are able to utilize insoluble fiber substrates begin to breakdown the complex fibers and release smaller, more soluble substrates for other bacterial groups, the secondary glycan degraders (McWilliam et al., 2007). Studies on the degradation of plant biomass substrates in the rumen have shown that bacterial groups that degrade readily fermentable substrates colonize the material first and are then replaced by groups that are better equipped to degrade the more recalcitrant substrates (Brulc et al., 2009) such as cellulose. The idea that insoluble dietary fibers and/or complex polysaccharide structures may provide a greater variety of fermentable substrates as different bacterial groups with different substrate specificity begin to degrade them, might explain the higher diversity found in the FMCs that fermented the mixtures of soluble and insoluble fibers as well as the changes in relative abundance (% of sequences) of the bacterial families over the 24-h in vitro fecal fermentation period. The decreases in diversity and changes in relative abundance of bacterial groups over time for the fiber substrates that were 100% soluble provide further proof of the effect of insoluble dietary fibers and/or complex polysaccharide structures on species diversity. Figure 5.12 shows that FOS does not support the proliferation of new bacterial families in the microbial community and, in a more discernible way, Quinoa SFF-86F predominantly favored the Bacteroidaceae family as percentages of Lachnospiraceae and Ruminococcaceae consistently decreased over the 24-h period and Bifidobacteriaceae was no longer detected after 6 h. On the other hand, the substrates that constituted mixtures of soluble-fermentable and insoluble-fermentable fibers promoted the growth of

bacterial families that were not detected on the original fecal microbiota. A 34.0% increase in the soluble-fermentable fiber for Quinoa M/ET-71F substrate resulted in the proliferation of the Veillonellaceae family after 12 h of fermentation. In the case of PMillet M/ET-82F, a 44.0% increase in insoluble-fermentable fiber promoted the growth of the Veillonellaceae family after 24 h of fermentation.

5.5 Conclusions

Although richness and diversity of FMCs did not change substantially, a decrease in diversity resulted from fermentation of FOS and Quinoa SFF-86F. In addition, all treated fiber substrates caused significant changes in the composition of the microbial communities. Substrates differing in composition, structure, and amounts of soluble-fermentable and insoluble-fermentable fiber promoted the growth of different bacterial groups. Based on specific changes observed, it was concluded that modification of pearl millet insoluble fibers with microwave and enzymatic treatments generated fiber substrates with significantly higher amounts of insoluble-fermentable fibers that caused increases in the abundance of the same bacterial families that were promoted by FOS. This was initially evidenced by the Bray-Curtis dissimilarity in which M/ET substrates from pearl millet clustered with FOS and confirmed by ANOSIM analysis. Pearl millet substrates, particularly after microwave+enzyme treatment, were more bifidogenic than quinoa. On the other hand, fiber substrates from quinoa clearly promoted the Ruminococcaceae family better than substrates from pearl millet and FOS. Time was also a determining factor in the compositional shifts of FMCs. The number of families detected increased with fermentation of fiber substrates containing fermentable soluble

and fermentable insoluble fibers and new families differed between untreated and microwave and enzyme-treated substrates per grain. Substrates that were 100% soluble had, in turn, resulted in a reduction of the species diversity in the microbial communities. These results suggest that the variety of polysaccharide structures that comprise the quinoa and pearl millet fiber substrates containing fermentable soluble and fermentable insoluble fibers create a microbial food chain in which the growth of a greater number of bacterial groups is supported. Besides the greater availability of a variety of polysaccharide structures arising from the treated fiber substrates, it is possible that after primary glycan degraders begin to breakdown the complex substrates available, other bacterial groups that are present at lower levels in the community may be promoted by the intermediate and end-products of glycan breakdown and fermentation. In addition, the effects of simple and easily fermentable carbohydrates resulted in the proliferation of specific groups that decreased diversity of the community. Thus, it is evident that shifts in microbiota can be achieved through use of a variety of fiber substrates. Perhaps combinations of them may be made according to specific changes that need to be effected within a gut microbiome.

Table 5.1 Dominant bacterial species in the microbial community from fecal samples used for in vitro fermentation

Phylum	Class	Order	Family	Species	% Abundance
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	**Lachnospiraceae**	**7.0**
				Blautia spp.	**16.0**
				Blautia obeum	0.9
				Blautia producta	0.2
				Coprococcus	**1.8**
				Dorea spp.	0.5
				Dorea formicigenerans	0.3
				Lachnospira	1.2
				Roseburia faecis	**2.7**
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	**Ruminooccaceae**	**5.3**
				Faecalibacterium prausnitzii	**9.8**
				Ruminococcus spp.	**1.6**
				Oscillospira spp.	0.7
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	**Bacteroides spp.**	**29.3**
				Bacteroides caccae	0.4
				Bacteroides eggerthii	0.6
				Bacteroides fragilis	0.1
				Bacteroides ovatus	0.7
				Bacteroides uniformis	**2.6**
Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	Bifidobacterium spp.	0.4
				Bifidobacterium adolescentis	0.2
				Bifidobacterium bifidum	0.2
				Bifidobacterium breve	**3.3**

Note: A total of 91 species were detected in the original fecal microbial community

Table 5.2 Two-way analysis of similarity (ANOSIM) tests for differences in fecal microbiota communities according to fiber substrates across all time points.

Group Pairing	R[a]	Significance Level %[b]
Quinoa TDF-49F & PMillet M/ET-82F	0.975	0.1
Quinoa TDF-49F & Quinoa M/ET-71F	0.938	0.1
Quinoa TDF-49F & FOS	1	0.1
Quinoa TDF-49F & PMillet TDF-51F	0.975	0.1
Quinoa TDF-49F & Quinoa MT-52F*NSD*	0.198	10.5
Quinoa TDF-49F & Quinoa SFF-86F	1	0.1
PMillet M/ET-82F& Quinoa M/ET-71F	0.975	0.1
PMillet M/ET-82F&FOS*NSD*	0.667	0.3
PMillet M/ET-82F& PMillet TDF-51F	0.975	0.1
PMillet M/ET-82F& Quinoa MT-52F	1	0.1
Quinoa M/ET-71F & FOS	0.988	0.1
Quinoa M/ET-71F & PMillet MT-51F	1	0.1
Quinoa M/ET-71F & Quinoa MT-52F	1	0.1
FOS & PMillet MT-57F	1	0.1
FOS & PMillet MT-51F	1	0.1
FOS & Quinoa MT-52F	0.988	0.1
FOS & Quinoa SFF-86F	1	0.1
PMillet-51F& Quinoa SFF-86F	1	0.1

[a] Global R-statistic = 0.883 [b] Bonferroni = 0.14%
[b] Significance ≤ 0.1 & $R \geq 0.883$
NSD Pairs compared are not significantly different.

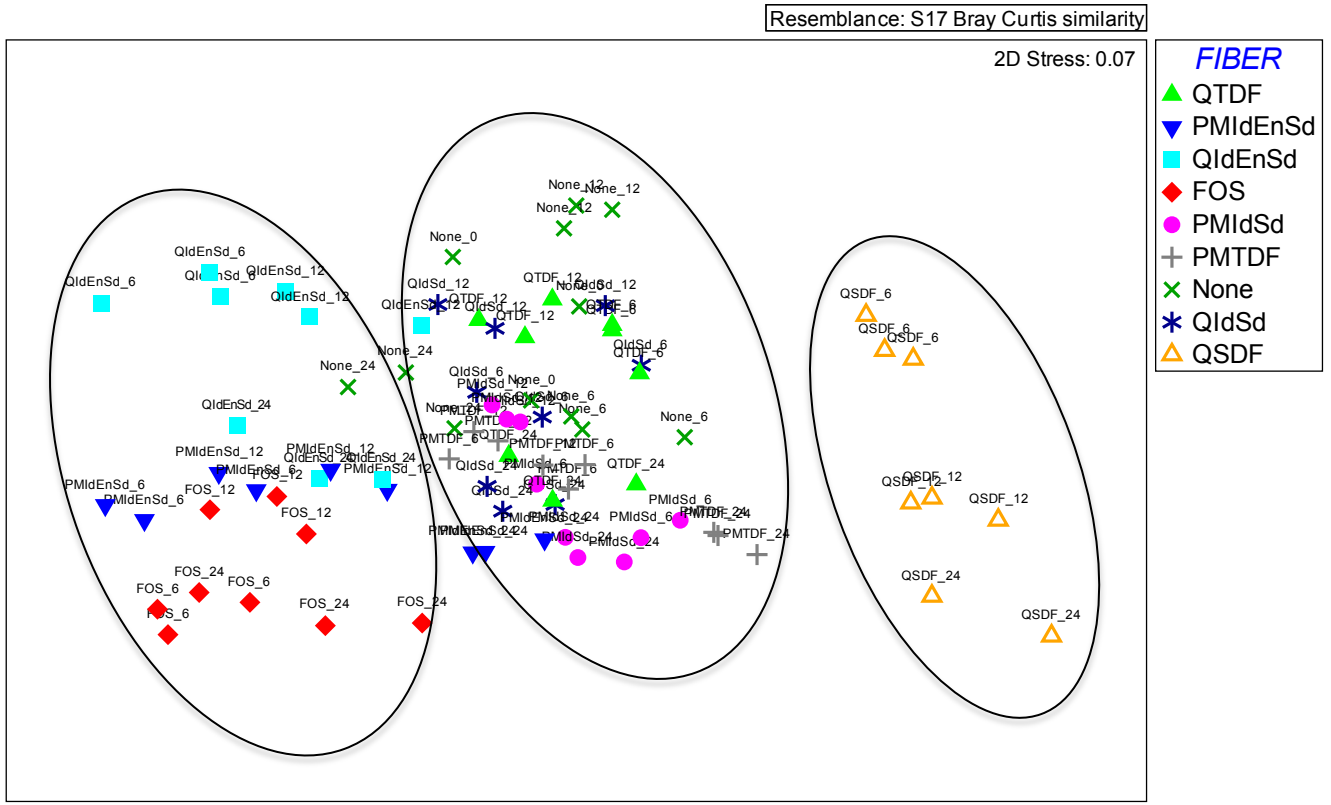


Figure 5.1 Bray-Curtis dissimilarity of fecal microbial communities after in vitro fermentation for 6, 12, and 24 h with treated fiber substrates. QTDF=quinoa untreated TDF (49% Fermentable), PMIdEnSd= microwave+enzyme-treated pearl millet substrate (82% Fermentable), QIdEnSd= microwave+enzyme-treated quinoa substrate (71% Fermentable), FOS = fructooligosaccharides (100% Fermentable), PMIdSd= microwave-treated pearl millet substrate (57% Fermentable), PMTDF= pearl millet untreated total dietary fiber substrate (51% Fermentablea), None=blank, no fiber added, QIdSd= microwave-treated quinoa substrate (71% Fermentable), QSDF=microwave-solubilized fiber from quinoa (100% Fermentable). Ovals depict the 3 different clusters in which the samples grouped.

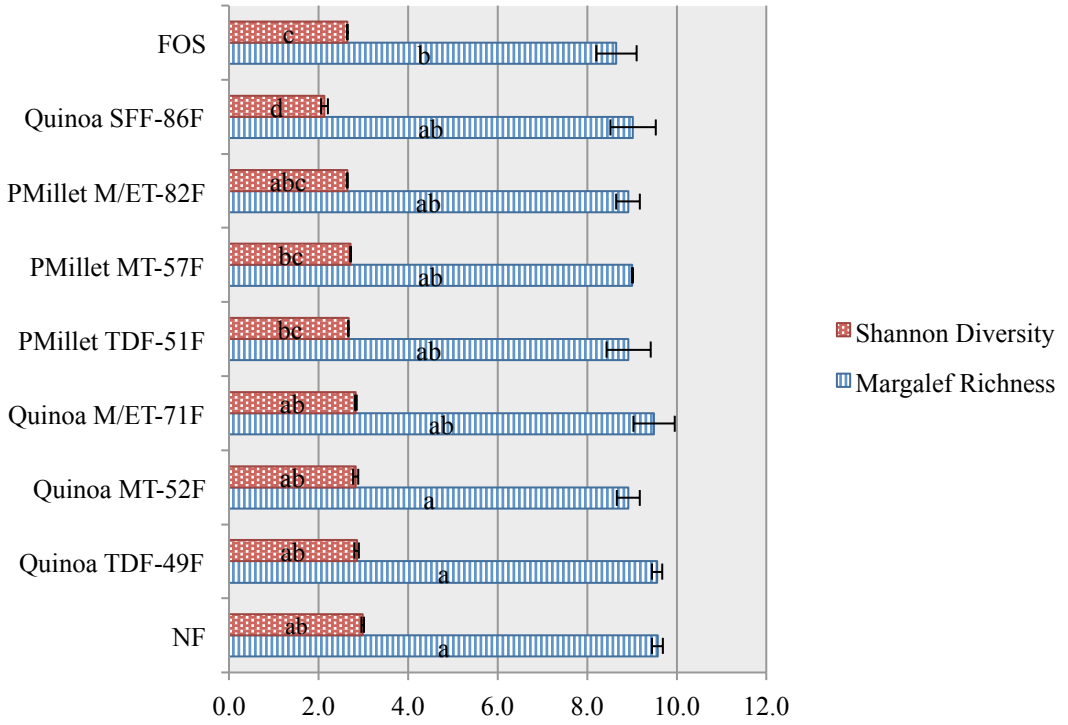


Figure 5.2 A-diversity indices, at species taxonomic level, of fecal microbiota communities after 24 h of in vitro fecal fermentation of treated fiber substrates from quinoa, pearl millet, and FOS. Values are the average of triplicate samples. Error bars represent standard deviation of replicate measurements. NF = blank, fecal samples with no added fiber substrate; Quinoa TDF-49F = untreated total dietary fiber from quinoa (49% fermentable); Quinoa M/ET-71F = microwave+enzyme treated fiber substrate from quinoa (71% fermentable); PMillet-51F = untreated total dietary fiber from pearl millet (51% Fermentable); PMillet M/ET-82F = microwave+enzyme treated fiber substrate from pearl millet (82% fermentable). Margalef Species Richness is a measure of the total # of species detected. Shannon Diversity Index is a measure that accounts for abundance and evenness of each species detected in the microbial community.

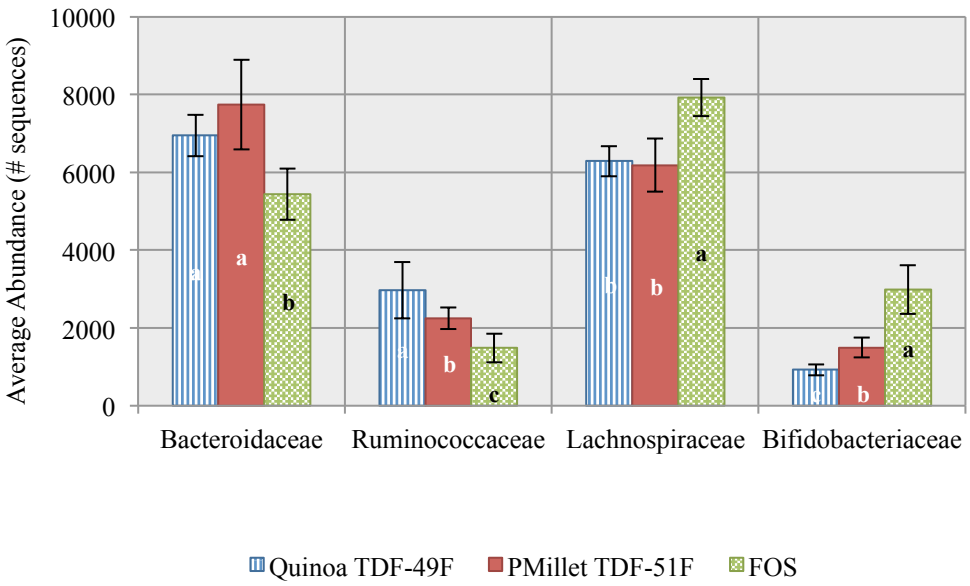


Figure 5.3 Average abundance (# of sequences) of the key differentiating families in fecal microbiota communities incubated with FOS, quinoa, and pearl millet TDF substrates across all time points. Genomic sequencing was performed on triplicate samples. Error bars represent the standard deviation of replicate measurements. Different letters within families indicate significant differences ($P<0.05$) between fecal microbial communities labeled by fiber substrate.

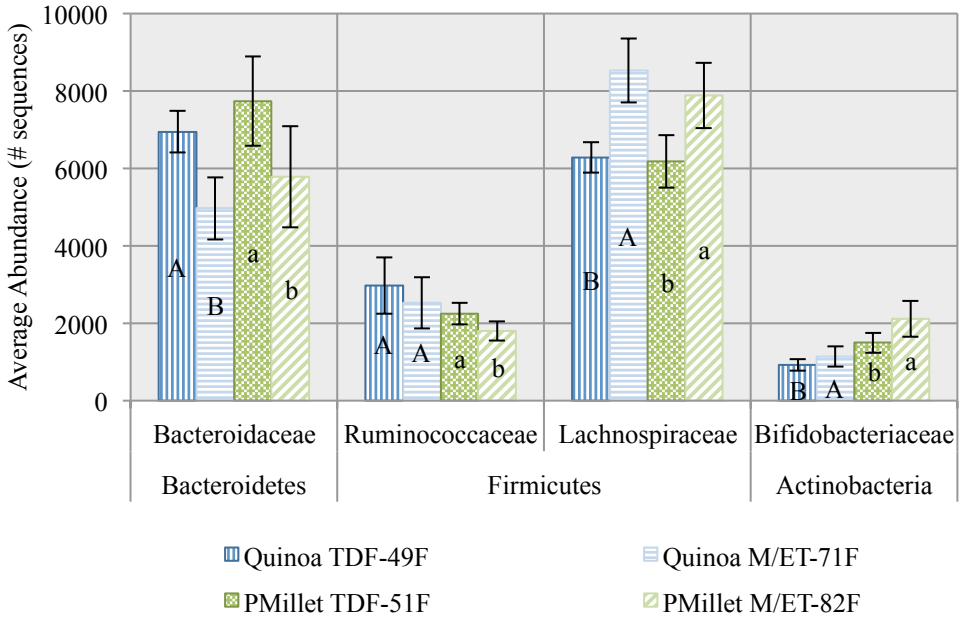


Figure 5.4 Average abundance (# of sequences) of the key differentiating families in fecal microbiota communities with TDF and M/ET substrates from quinoa and pearl millet across all time points. Genomic sequencing was performed on triplicate samples. Error bars represent the standard deviation of replicate measurements. Different letters within families indicate significant differences ($P<0.05$) between fecal microbial communities labeled by fiber substrate; capital letters indicate differences between quinoa substrates, small letters indicate differences between pearl millet substrates.

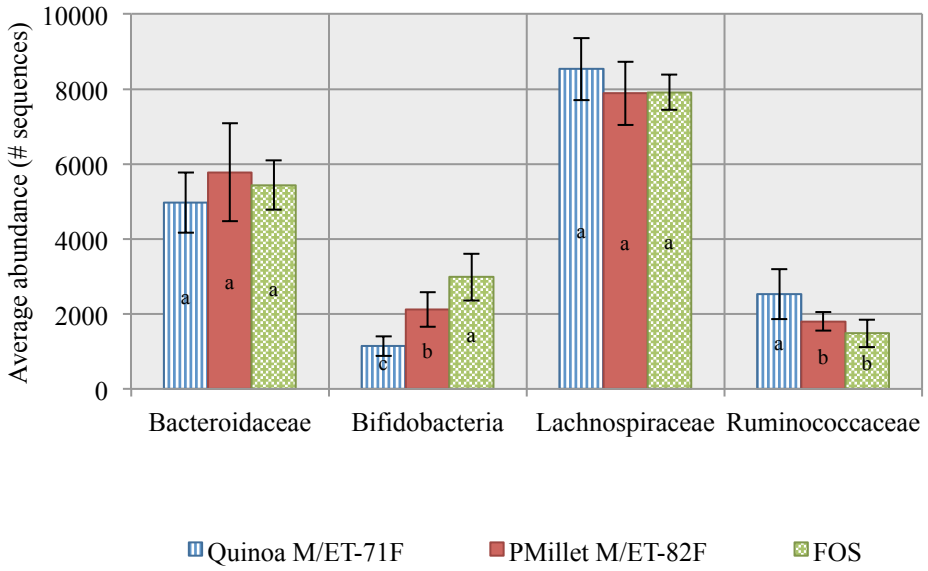


Figure 5.5 Average abundance (# of sequences) of the key differentiating families in fecal microbiota communities incubated with FOS and M/ET substrates from quinoa and pearl millet across all time points. Genomic sequencing performed on triplicate samples. Error bars represent standard deviation of replicate measurements. Different letters within families indicate significant differences ($P<0.05$) between fecal microbial communities labeled by fiber substrate.

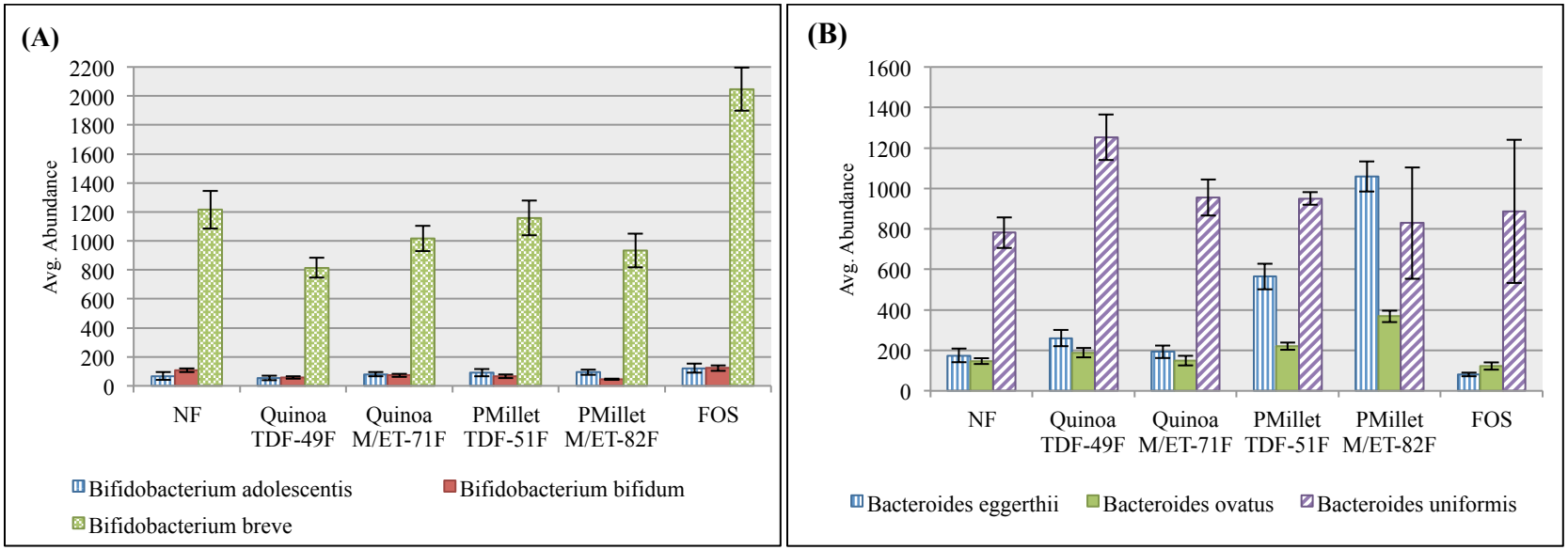


Figure 5.6 Predominant bacterial species by family in fecal microbiota communities after in vitro fermentation for 24 h. (A) Dominant species belonging to Bifidobacteriaceae family. (B) Dominant species belonging to Bacteroidaceae family. Genomic sequencing performed on triplicate samples. Data are presented as Average Abundance (# of sequences). Error bars represent standard deviation of triplicate measurements. NF = blank, fecal samples with no added fiber substrate; Quinoa TDF-49F = untreated total dietary fiber from quinoa (49% fermentable); Quinoa M/ET-71F = microwave+enzyme treated fiber substrate from quinoa (71% fermentable); PMillet TDF-51F = untreated total dietary fiber from pearl millet (51% Fermentable); PMillet M/ET-82F = microwave+enzyme treated fiber substrate from pearl millet (82% fermentable).

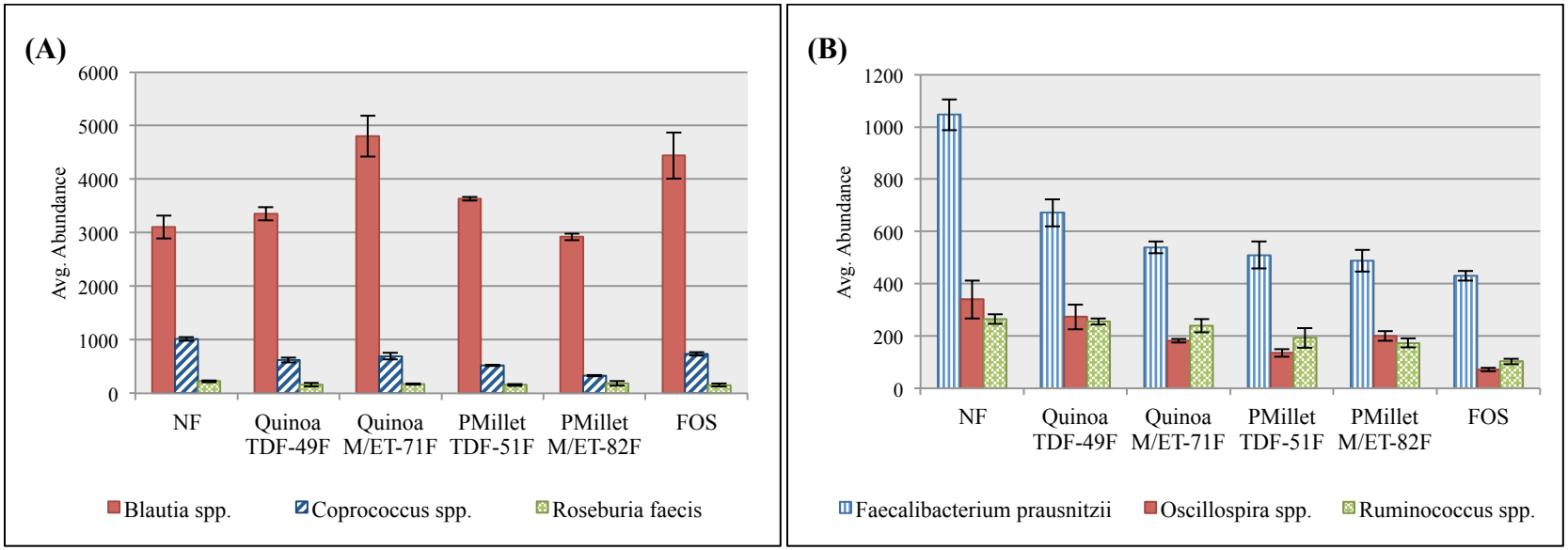


Figure 5.7 Predominant bacterial species by family in fecal microbiota communities after in vitro fermentation for 24 h. (A) Dominant species belonging to Lachnospiraceae family. (B) Dominant species belonging to Ruminococcaceae family. Genomic sequencing performed on triplicate samples. Data are presented as Average Abundance (# sequences). Error bars represent standard deviation of triplicate measurements. NF = blank, fecal samples with no added fiber substrate; Quinoa TDF-49F = untreated total dietary fiber from quinoa (49% fermentable); Quinoa M/ET-71F = microwave+enzyme treated fiber substrate from quinoa (71% fermentable); PMillet TDF-51F = untreated total dietary fiber from pearl millet (51% Fermentable); PMillet M/ET-82F = microwave+enzyme treated fiber substrate from pearl millet (82% fermentable).

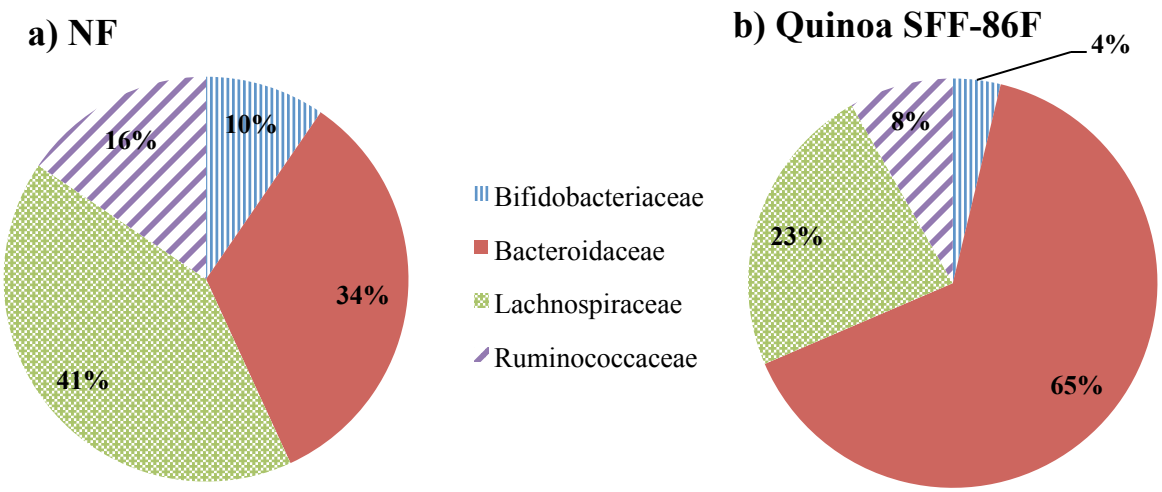


Figure 5.8 Percent abundance of the four key differentiating families that constitute the fecal microbiota communities of (A) Blank-No Fiber samples, and (B) Quinoa SFF-86F sample. Data is presented as the % of total abundances that belong to each family.

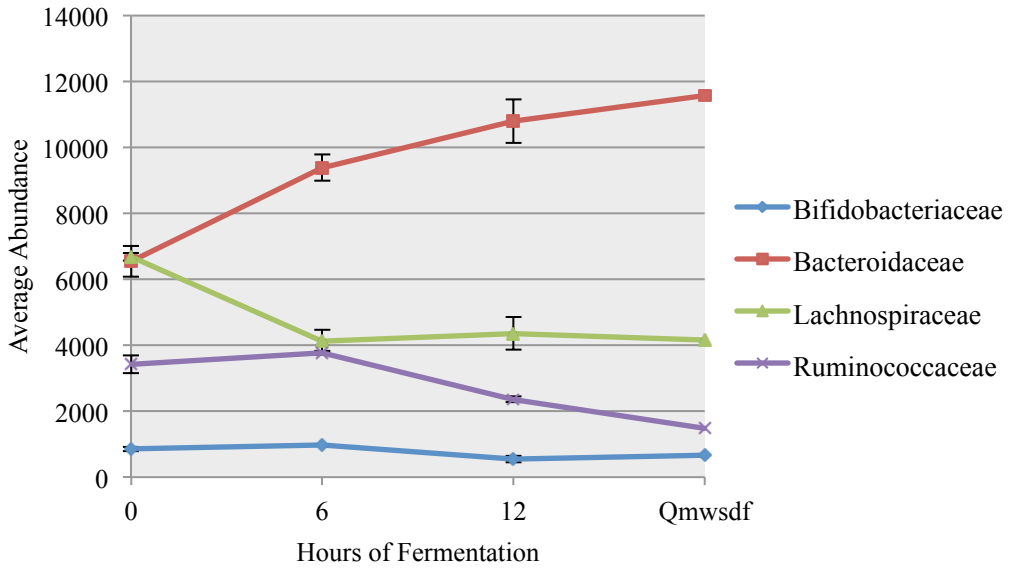


Figure 5.9 Changes in average abundance of each major bacterial family in the fecal microbiota community from fermentation of Quinoa SFF-86F over the 24 h fermentation period. Data is presented as the average abundance (# sequences) of triplicate samples. Error bars represent standard deviation of replicate measurements.

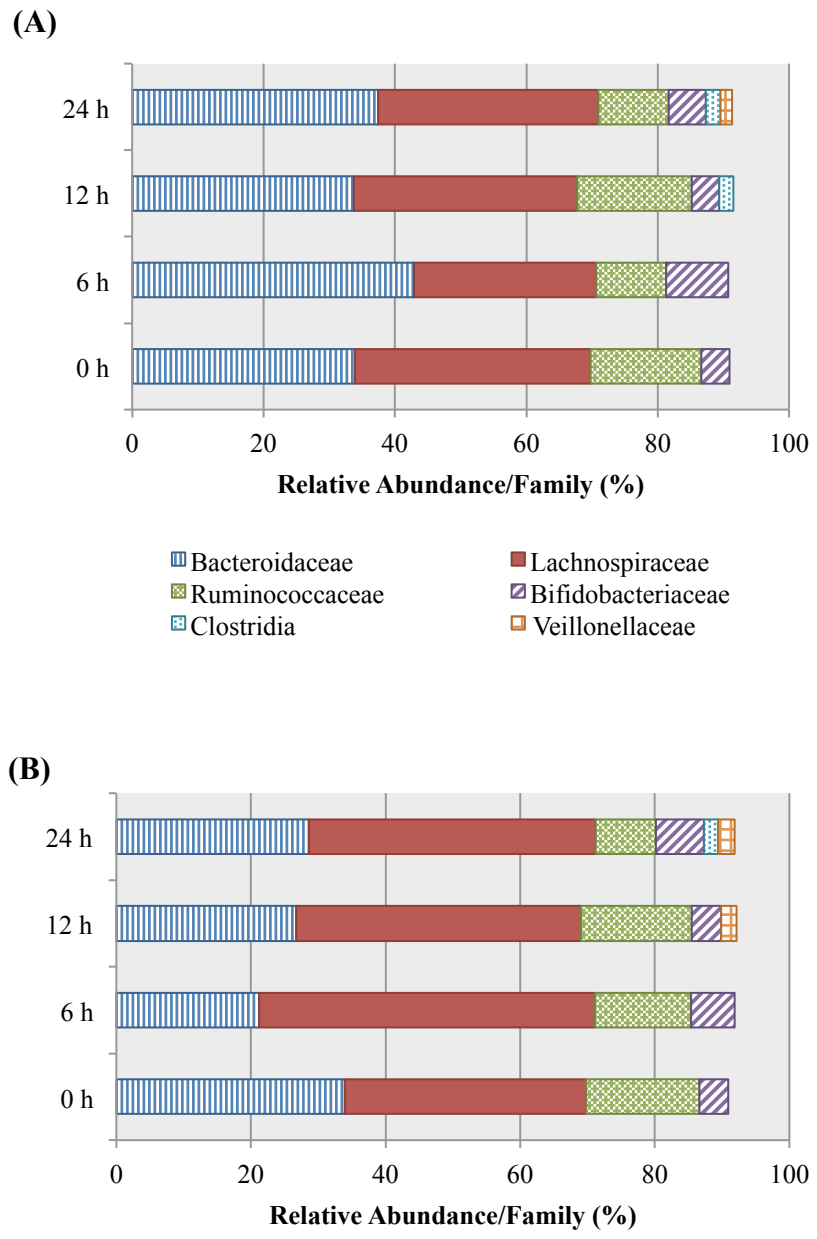


Figure 5.10 Changes in the relative abundance (% of sequences) per family in fecal microbiota communities of (A) Quinoa TDF-49F, and (B) Quinoa M/ET-71F fiber substrates over the 24 h in vitro fermentation. The sum of percentages does not add to 100% because of a portion (~9%) of sequences that were not assigned to a specific family.

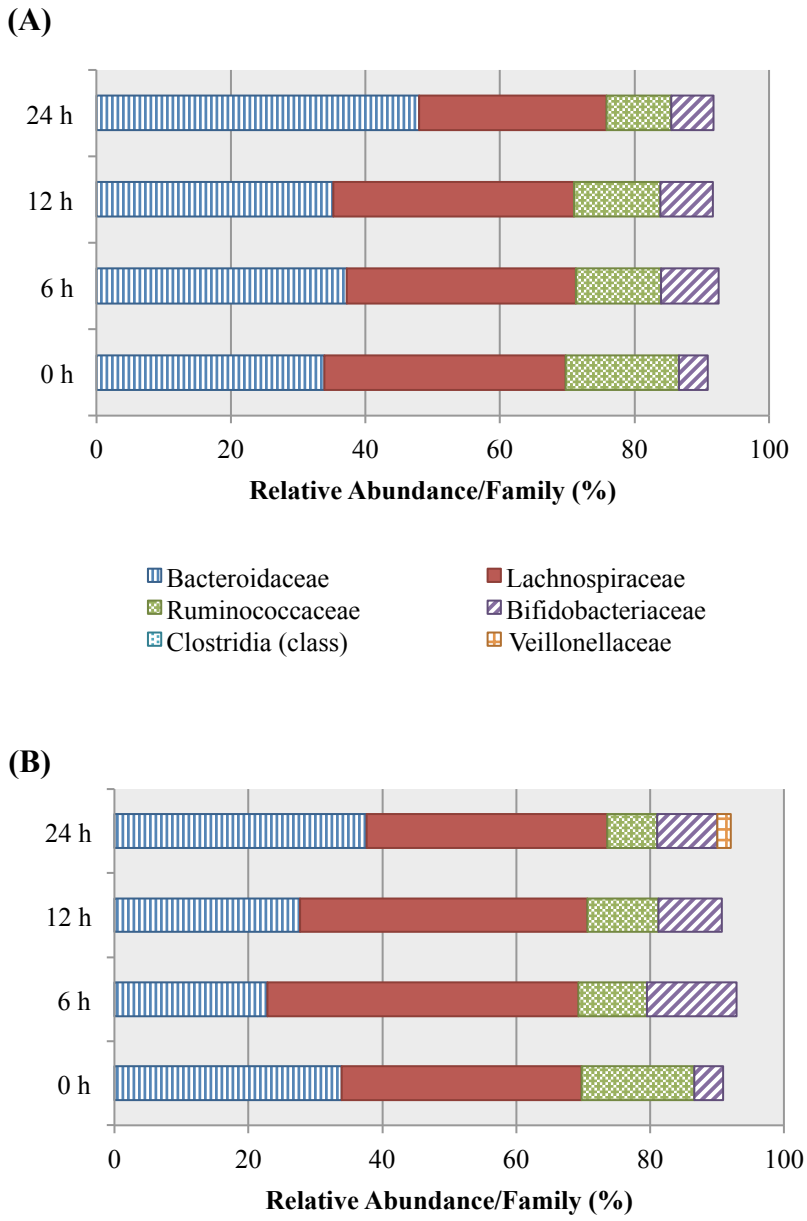
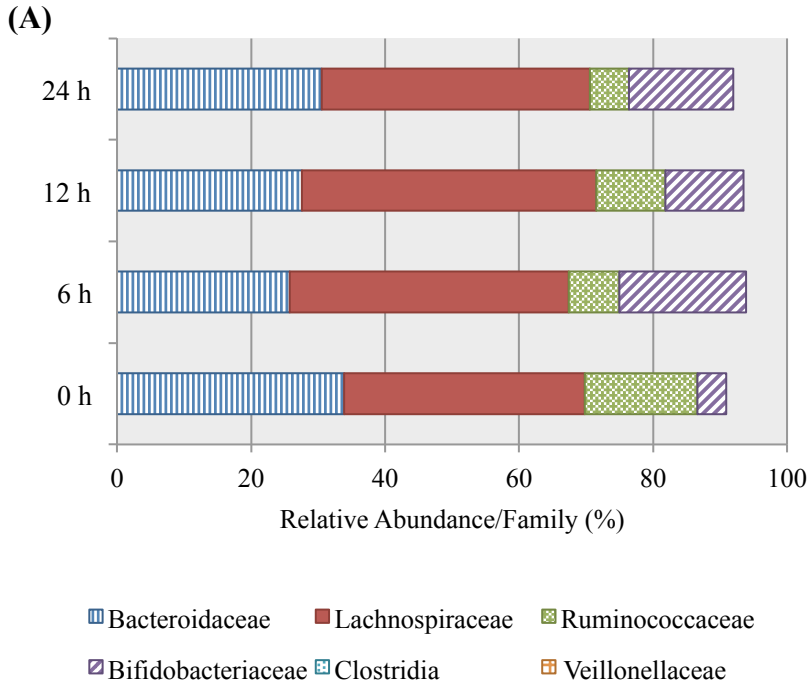


Figure 5.11 Changes in the relative abundance (% of sequences) per family in fecal microbiota communities of (A) PMillet TDF-51F, and (B) PMillet M/ET-82F over the 24 h period of in vitro fermentation. The sum of percentages does not add to 100% because of a portion (~9%) of sequences that were not assigned to a specific family.



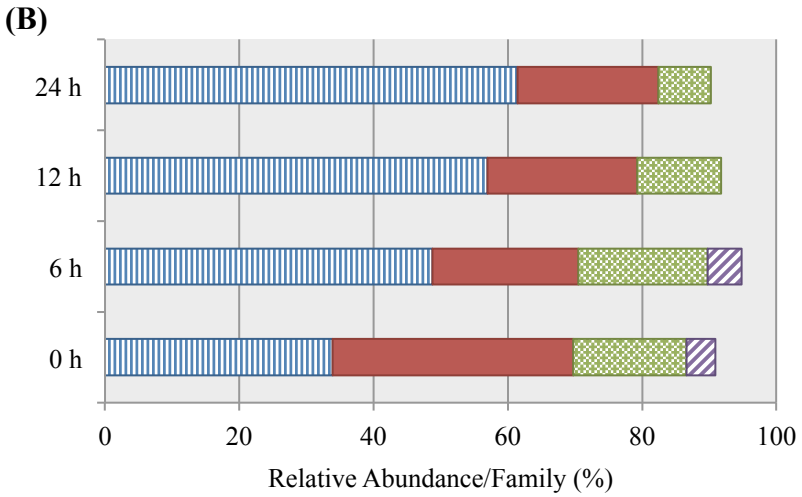


Figure 5.12 Changes in the relative abundance (% of sequences) per family in fecal microbiota communities of (A) FOS, and (B) Quinoa SFF-86F over the 24 h period of in vitro fecal fermentation. The sum of percentages does not add to 100% because of a portion (~9%) of sequences that were not assigned to a specific family

OVERALL CONCLUSIONS AND FUTURE WORK

The study of the effects of dietary fiber in the gut is of importance because the metabolites produced from its fermentation by gut microbes are key regulators of their symbiotic relationship with the host and, consequently, of the host's health and susceptibility to disease. In this research project, dietary fibers from alternative grains were investigated before and after hydrothermal and enzymatic treatments for the generation and effect of fermentable carbohydrate substrates. *In vitro* fecal fermentation profiles and effects on the composition of fecal microbiota community were evaluated. Dietary fibers were quantified and isolated from four alternative grain sources - sorghum, pearl millet, quinoa, and amaranth. Both cereal and pseudocereal grains contained comparable amounts of total dietary fiber (9.3 to 11.4%). A greater portion of the fiber from quinoa and amaranth was soluble (22.0% pseudocereals vs. 13.0-15% cereals). Being dicots, composition of the insoluble and soluble dietary fibers from the pseudocereals differed from that of the cereals. Insoluble fiber from quinoa and amaranth was composed of pectic substances namely, homogalacturonans and rhamnogalacturonan-I with arabinan side-chains, highly branched xyloglucans, cellulose, and lignin. Insoluble dietary fiber from sorghum and pearl millet was composed of lignin, cellulose, and arabinoxylans. In the case of soluble dietary fibers, xyloglucans made up to ~40.0-60.0% and arabinose-rich pectic polysaccharides represented ~34.0-55.0% in

quinoa and amaranth. For sorghum and pearl millet, the soluble fiber fraction was mainly composed of mannose and glucose. Their glucomannan content was low, and they also contained a small amount of highly branched AXs. The insoluble dietary fiber fractions were subjected to hydrothermal and enzymatic treatments to effect solubilization and improve their fermentability through the soluble fermentable and insoluble fermentable fractions. Overall, the treatments caused marked difference in composition of the dietary fibers and different fermentation effects. Pseudocereals were more susceptible to solubilization by the combination of the hydrothermal treatments used in this study and enzymatic treatments resulting in 32.9-53.9% soluble fiber from IDF, most likely due to lower inherent amount of lignin. Cereal samples did not respond as well, with only ~13.0-21.0% maximum solubilization achieved from treatment of the IDF. Microwave radiation treatments in combination with enzymatic hydrolysis of insoluble dietary fibers from quinoa and pearl millet resulted in a range of treated fiber substrates (mixed insoluble/soluble fiber preparations) that contained a variety of oligosaccharides differing in composition and structure. Highly branched soluble oligosaccharides, mainly arising from arabinoxylans, were generated from pearl millet insoluble dietary fiber. In the case of quinoa, soluble oligosaccharides from cellulose and pectic polysaccharides were made in comparable amounts.

Solubilization of insoluble dietary fibers appeared to be promising in generating pectic, glucan and xylo-oligosaccharides with potentially desirable fermentable and prebiotic properties. Accordingly, the fermentability of the treated fiber substrates was evaluated using in vitro human fecal fermentation. Increases in gas and SCFA production as well

as shifts in SCFA profiles indicated improved fermentation profiles of the insoluble dietary fibers due to treatments. Although soluble fiber content of treated substrates from pearl millet was significantly lower (~20%) than for quinoa, its fermentability was on par with it indicating that its insoluble fraction was made more fermentable. Still, differences in initial rate of gas production were observed. Pearl millet fiber substrate had a lower initial rate of gas production than quinoa owing to its lower amount of soluble fermentable fiber. Thus, improvements in fermentability were not entirely due to increases in fiber solubility and provide a new approach for the development of fermentable carbohydrate substrates, as researchers tend to mainly focus on soluble fibers.

Furthermore, the treated fiber substrates with increased fermentability caused significant shifts in the fecal microbiota community. The relative abundance, at family and species levels, of bacterial groups changed according to type of fiber and time of fermentation. Treated fiber substrates derived from quinoa insoluble dietary fiber promoted the Ruminococcaceae family better than substrates derived from pearl millet or FOS, but pearl millet substrates were more bifidogenic than those from quinoa. The combination of soluble-fermentable substrates with insoluble-fermentable carbohydrate polymers supported the growth of a larger of number of bacterial groups than FOS. Thus, insoluble dietary fibers that are found in high amounts in the by-products of cereal (or pseudocereal) milling, can be treated and effectively used as sources of fiber substrates with increased fermentability and leading to high microbiota complexity that may have potential health-benefitting properties.

Future work on the treated fiber substrates should focus on thorough characterizations of the fermentable fiber fractions that were generated by the treatments, as this will provide a better understanding of the resulting fermentation profiles and microbiota changes. Moreover, in-depth bioinformatics analysis will provide detailed information on how the composition and fermentability of each fiber substrates relates to the specific bacterial groups whose relative abundances shifted significantly. This information may shed light on the complexity of dietary fiber fermentation in the gut and provide more insight about how gut microbiota modulation can be achieved with fermentable fibers.

In terms of potential applications, an evaluation of the functional properties of the fermentable fiber fractions may be performed as well as trials for their incorporation into processed foods, analysis of their effects on organoleptic properties, and/or for the development of fiber supplement products. As more information on the fermentative and functional properties of these fermentable fibers is gathered, an optimization and scale-up of the processing techniques used here should be performed. An optimization of the techniques may also provide a way to design different treatment combinations in order to obtain fiber substrates with specific properties.

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APPENDICES

APPENDICES

A) Results

Table A.1 Description of steps to enzymatically isolate insoluble dietary fibers from whole grain flours.

Step	Description
#1 Defatting whole grain flour (WGF)	a) 100-200 g of WGF are suspended in hexane (1:7, w/v). ▪ Pseudocereals: 90 min ▪ Cereals: 60 min b) Hexane was removed by filtration and air-drying overnight.
#2 Enzymatic removal of starch & protein	a) Defatted WGFs suspended in water (1:10, w/v) were mixed with thermostable α-amylase. b) Mixture is heated 90 °C with constant stirring. After 2 hours, a 2nd dose of α-amylase is added, incubated for a total of 6 hours, and filtered to remove liquefied starch. c) WGF slurry is re-suspended in water (1:10, w/v), heated to 50 °C and incubated with protease for 4 hours (protease is inactivated by boiling slurry for 15 min with constant stirring).
#3 Fiber fractions separation	a) WGF slurry if filtered and insoluble dietary fiber residue y re-suspended in water (1:10, w/v). b) Filtrate washings containing soluble fiber fraction are collected in a clean container.
#4 2nd enzymatic removal of starch and protein	a) Amylase and protease incubations are repeated on suspension of insoluble dietary fiber as described above (steps #2a→c). b) Filtrate washings containing soluble fiber fraction are incubated with amyloglucosidase, dialyzed (MWCO 12-14 kDa) and freeze-dried.
#5 Insoluble fiber washings, drying & grinding	a) Insoluble fiber residue are collected by filtration and washed with fresh water twice followed by 80% aqueous ethanol. b) This washing step was repeated once. c) Collected fiber is dried at 50 °C in a convection oven overnight. d) Dried fiber is then ground to a fine powder with cyclone mill.

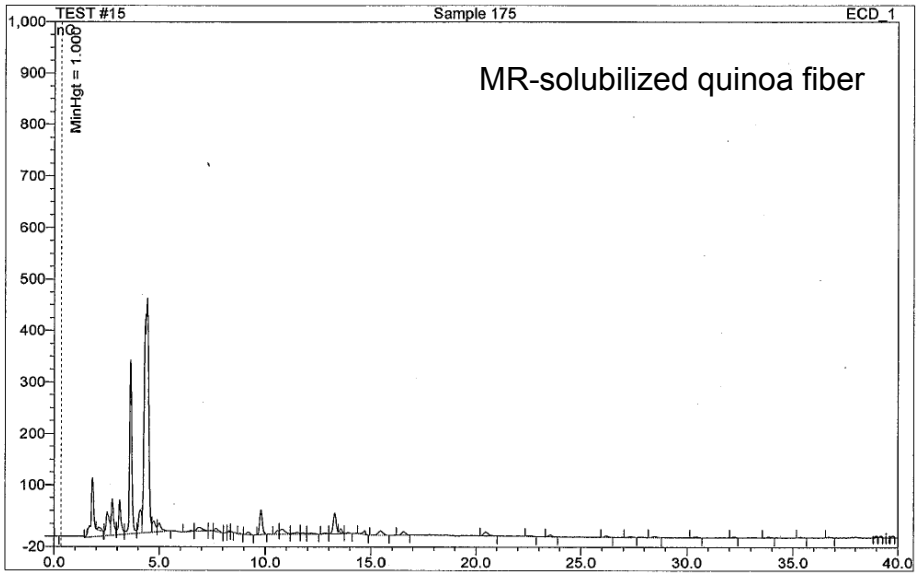

Figure A.1. HPAEC chromatogram of MR-solubilized fiber from quinoa.

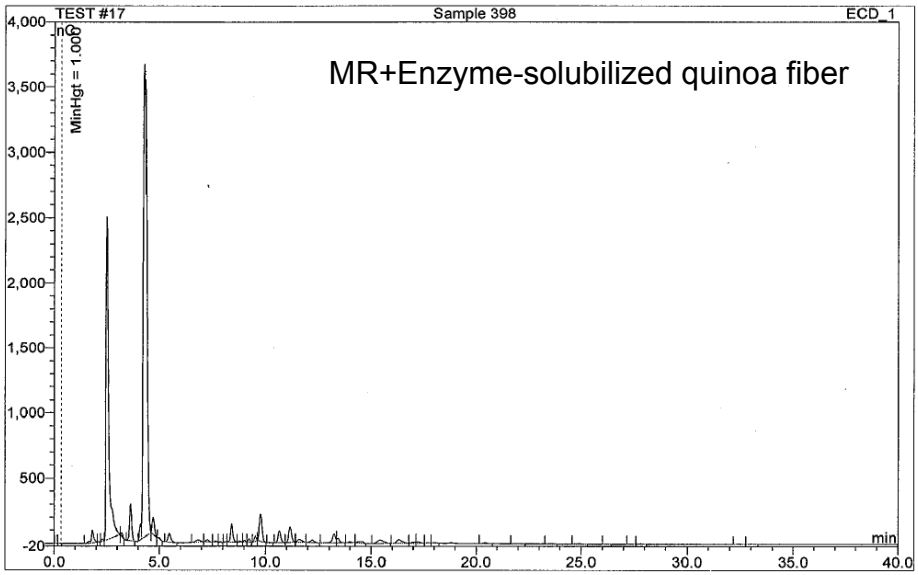

Figure A.2. HPAEC chromatogram of MR+enzyme-solubilized fiber from quinoa.

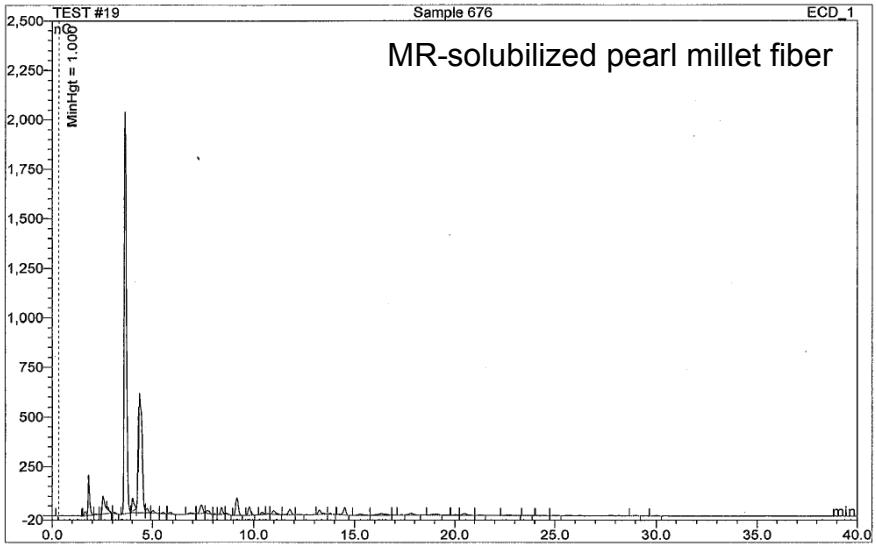

Figure A.3. HPAEC chromatogram of MR-solubilized fiber from pearl millet.

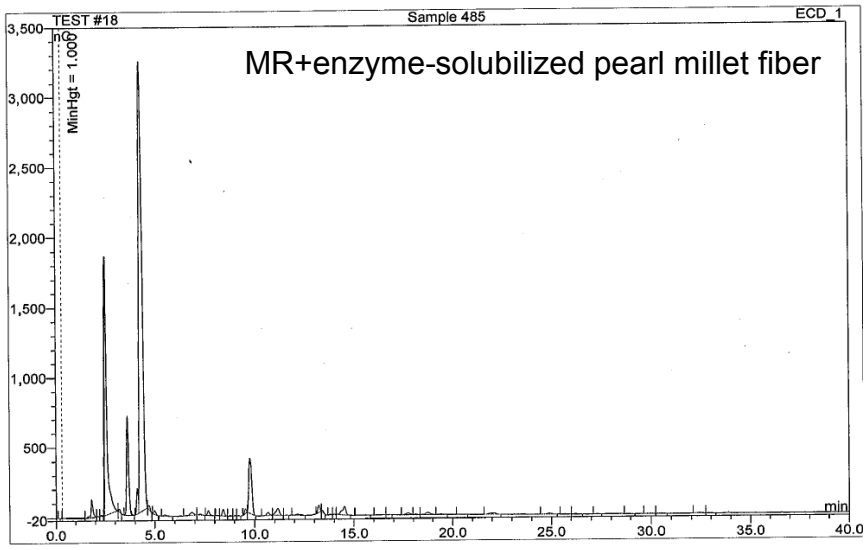

Figure A.4. HPAEC chromatogram of MR+enzyme-solubilized fiber from pearl millet.

B) Procedures

B.1 Calculation of Fermentable Fiber in Treated Fiber Substrates

1) Fermentable fiber content for each treated fiber substrate was calculated based on the sum of acetate, propionate and butyrate production after 24 h of in vitro fecal fermentation. Gas and other fermentation end products were not accounted for in the calculations because composition of the gas produced was unknown and amounts of lactate were negligible (data not shown here). Fermentable fiber contents were calculate as follows:

$$\left(\mu\text{Mol SCFA} \times \frac{\text{Mol}}{\mu\text{Mol}}\right) \times \frac{\text{g}}{\text{Mol}} = \text{g SCFA}$$

$$\frac{\text{g SCFA}}{\text{g Treated Fiber}} \times 100 = \text{Total Fermentable Fiber (}\mathbf{TFF}\text{)}$$

Soluble-Fermentable Fiber (**SFF**) = % Soluble Fiber Content

Insoluble-Fermentable Fiber (**IFF**) = TFF – SFF

Insoluble-Nonfermentable Fiber (**INFF**) = 100-TFF

VITA

VITA

Lisa M. Lamothe received her B.S. degree in Food Science and Technology from Zamorano University in Honduras. She then received her M.S. degree in Food Science from Purdue University under the advisement of Bruce Hamaker and her research focused on the development of screening methods for breeder selection of popcorn hybrids. After her obtaining her M.S. degree, she worked for Cargill Meats Central America in Honduras as a member of the Quality Assurance & Food Safety team. For the past 3.5 years, Lisa has been working on her Ph.D. degree in Food Science at Purdue University, under the same advisor, and her work focused on the generation of fermentable carbohydrate substrates for improved colonic health from insoluble dietary fibers. After receiving her Ph.D., she will begin an Associate Research Position at the Nestlé Research Center in Lausanne, Switzerland.

PUBLICATIONS

PUBLICATIONS

Lamothe, L. M., Srichuwong, S., Reuhs, B. L., and Hamaker, B. R. 2015. Quinoa (Chenopodium quinoa W.) and amaranth (Amaranthus caudatus L.) provide dietary fibers high in pectic substances and xyloglucans. Food Chemistry, 167:490-496

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